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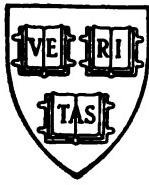
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**THE JOURNAL OF  
EXPERIMENTAL MEDICINE**



# THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

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WITH SEVENTY-SEVEN PLATES AND FORTY-EIGHT  
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# DOES CARDIAC RHYTHM ALONE DETERMINE HUMAN BLOOD PRESSURE VARIATIONS?\*

By CARL J. WIGGERS, M.D.

(*From the Physiological Laboratory of Cornell University Medical College,  
New York.*)

PLATES 1 TO 6.

## INTRODUCTION.

The observation that blood pressure undergoes periodic variations with inspiration and expiration dates from the experiments of Stephen Hales (1) in 1733. Their nature and causes, though frequently reinvestigated, still remain subjects for physiological discussion. One phase of the question, the part played by variations in cardiac rate, is again considered in this investigation.

An extensive literature has grown about this question. Ludwig (2) observed these variations, placed the time of acceleration in the expiratory period, and disregarded its possible significance in the causation of pressure variations. It was held in similar disregard by Magendie (3), as well as by Donders (4). It remained for Ludwig's pupil Einbrodt (5) to show that in dogs the pressure rises in inspiration and falls in expiration. The increase in heart rate during inspiration, together with an increased blood flow through the thorax, was held to account for the inspiratory rise of pressure. Einbrodt, however, considered the first as of distinctly minor importance, for, after a rhythmic condition of the heart had been produced by vagus section, the blood pressure variations were frequently even increased.

Burden-Sanderson (6) confirmed the observation that the heart rate increased during inspiration. He believed that the enlargement of the thorax caused a belated expansion of the veins and the relaxing heart, which favored their filling. He reasoned that the more rapidly the cavities filled, the shorter the period of relaxation, the more vigorous its systole, and hence the higher the pressure.

Kowalewsky (7) and Zuntz (8) mention the variations of cardiac rate as contributing to the periodic blood pressure variations. François-Franck (9), Sommerbrodt (10), and Schweinburg (11) have made no reference to them in this connection.

Fredericq (12) demonstrated that probably these variations were dependent on a central influence and clearly analyzed the part they played in respiratory variations of blood pressure. He pointed out that variations of blood pressure occurred in the dog with respiratory phases, whether the heart was rhythmic or arrhythmic. There was this difference: in the former case blood pressure fell

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## **2      Does Cardiac Rhythm Determine Blood Pressure Variations?**

during inspiration, in the latter it rose. He also pointed out that in animals where no normal variation in cardiac rate occurred (rabbit), the pressure always fell during inspiration. Fredericq, therefore, concluded that the mechanical influence of respiration tended to reduce arterial pressure during inspiration, but that in the dog these changes were overbalanced by an acceleration of the heart. Inasmuch as he observed a similar pulse variation in himself, he concluded that cardiac rhythm determined the direction of human blood pressure.

Various investigators, Riegel (13), Landois (14), Schreiber (15), Sommerbrodt (10), and others, using the sphygmograph as an indicator of blood pressure changes, concluded that, in man, a fall of pressure and not a rise occurred during inspiration. In 1886, however, Klemensiewicz (16) showed by simultaneous graphic records of respiration that the main change was a rise during inspiration which was preceded by a fall if cardiac acceleration was delayed in its onset. Werthheimer and Meyer (17) confirmed this and showed that atropin not only prevented the inspiratory cardiac acceleration but also the consequent rise in pressure. A glance at the published records of these investigators shows that only the lowest level of each pulse wave rises while the upper portion distinctly falls during inspiration. Such records tend to show that the cardiac rate determines the variation in diastolic pressure. Sphygmograph tracings taken from different individuals by other investigators, however, show both systolic and diastolic portions to vary as frequently in the same direction. While some of these variations may be attributed to individual variation, the point has been raised that the systolic and diastolic portions of sphygmograph records may not be regarded as a reliable index of the trend of pressure variations on account of the varying volume of the *venæ comites* (Hill, Barnard, and Sequeira (18), and Hirschman (19)).

More recently Lewis (20) attempted to obviate these objections against the use of the instrument as a pressure indicator by suspending it from the wrist. From seventeen experiments upon this point, he concluded that the most frequent type of pressure variation during normal breathing is due to heart rate variation, systolic and diastolic pressure changing in the opposite directions as the heart slows and accelerates. During deep thoracic or abdominal breathing, however, another form of variation is added which is due to the mechanical influence of respiration.

Last year Erlanger and Festerling (21) showed that "the size of the sphygmomanometer oscillations" (*i. e.*, diastolic pressure) "was not materially influenced by such pulse pressure changes as probably result from the changing systolic output," and further, that when breathing rate was voluntarily altered they followed the variations of respiration and not heart rate. A similar conclusion was arrived at by the mathematical analysis of Frank (22) who concluded that "moderate changes in the normal cardiac frequency can cause no change in blood pressure, since it already lies near the maximum." The writer (23) tested this question in the pulmonary circuit with the result that, within the range of cardiac cycle variation most commonly found in man (0.60 to 0.90), the length of the cardiac cycle was practically without influence on systolic and diastolic pressures as measured by an improved pulse pressure instrument.

This review establishes the idea that great variation in cardiac rate may determine entirely the trend of blood pressure changes.

It is generally admitted, however, that, in addition, there exists a mechanical influence due to respiratory movements which tends to exert a contrary influence on blood pressure and predominates when heart rate changes are not marked. It is not entirely agreed, however, whether the cardiac variations in man are great enough to exert any influence counteracting the effect of respiration itself, or whether there are individual variations.

Opposed to the current view as to the part played by respiration is that given in a recent communication by Henderson and Barringer (24). These investigators hold that it is impossible for the mechanical influence of respiration to modify arterial pressure except as it operates through a change in heart rate. Briefly stated their position is as follows: venous pressure in man and animals is always above a certain critical value, and therefore all beats of the mammalian heart are superimposable. This condition, however, is difficult to maintain in experimental animals and most experiments are performed with the venous pressure below the critical level. In such cases the most that respiration can do is to modify ventricular filling by periodically raising the venous pressure to a critical level, and arterial pressure may be modified independently of the rate. By special precautions (induction of hypercapnia and saline infusion), however, it is possible to preserve a critical pressure and reduplicate normal conditions. In such cases the systolic output and arterial pressure are determined solely by the length of the previous diastole. Henderson would harmonize the discordant observations as to whether the human pressure rises or falls in inspiration by saying that the change in cardiac rhythm bears no constant relation to these respiratory phases.

As this view rests upon experimental facts supporting the hypothesis that in normal animals it is physiologically impossible for mechanical influences of respiration to modify the blood pressure apart from changes in the heart rate, it becomes desirable to reinvestigate the question of how closely systolic and diastolic pressures in man vary with the duration of the previous heart cycles.

#### THE ESTIMATION OF HUMAN PRESSURE VARIATIONS BY THE SPHYGMOSCOPE OSCILLATIONS.

##### ANALYSIS OF PREVIOUS WORK.

The methods for detecting variation of human blood pressures have not been entirely satisfactory. They have been recently reviewed by Erlanger and Festerling (21) who, among other procedures, suggested a convenient method which may be briefly described as follows: The cuff of an Erlanger blood pressure apparatus was applied to an arm and the systolic pressure estimated. The pressure was then reduced five or ten millimeters, at which level a continuous record of pulsations, with the tambour attached to Erlanger's instrument, was recorded.

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The method rests upon the principle that "if a pressure lying anywhere between systolic and diastolic be applied to an artery through a recording sphygmomanometer adapted to employ the principle of Marey, an oscillation is recorded whose amplitude is larger than that obtained at systolic pressure and smaller than that at diastolic pressure. If, while this pressure on the artery is maintained, . . . the intra-arterial minimum pressure should approach the extra-arterial pressure, the amplitude of the recorded waves should, as a result, increase; and *vice versa*, . . ."

It was, of course, evident to these investigators that the principle of Marey could be theoretically applied in this fashion to pressure measurements only provided that the pulse pressure were constant during consecutive beats of the heart, for upon the amplitude in pulse pressure the oscillations primarily depend. They, therefore, carried out animal experiments to demonstrate not only the validity of the theory that the amplitude of oscillations depended on the relation of extra-arterial to intra-arterial minimal pressure, but sought also to determine what modifying effect variations of pulse pressure exerted. Their conclusions on this point, here consecutively stated, were: (a) "Ordinarily, however, as experiments on animals demonstrate, pulse pressure changes do not mask blood pressure changes"; (b) "Nevertheless, one should keep in mind the danger of drawing conclusions from continuous blood pressure records without, at the same time, taking into consideration all of the factors at work in producing the changes";<sup>1</sup> and (c) in man, "the height of the oscillations is not materially influenced by such pulse pressure changes as probably result from the changing systolic output. . . ."

The experimental data, however, do not seem to establish satisfactorily the fact that these inferences may also be applied in cases where variation in the length of cardiac cycle is responsible for pulse pressure changes.

In the first place the records submitted show no satisfactory evidence that variations in diastolic pressure due to cardiac rhythm cause corresponding variations in the sphygmomanometer oscillations which may not as well be explained by pulse pressure variations or by the fact that the extra-arterial pressure was obliterating the vessel within the arteriograph. A case is pointed out by one of the authors (25) as bearing upon this point. For example, the systolic pressure

<sup>1</sup> Erlanger, J., and Festerling, E. G., *loc. cit.*, p. 381.

of the last vagus pulse of the second vagus stimulation in figure 3 in the article of Erlanger and Festerling rises almost to the same systolic level as the pulse following. The diastolic pressure of the former is far below that of the latter, yet despite the great difference in pulse pressure in favor of the vagus beat, the sphygmomanometer oscillations are distinctly smaller. To judge from the impact waves of the sphygmomanometer, however, the artery was entirely collapsed within the arteriograph during the vagus beat and not during the beat following, in which case no correspondence could be expected. Unquestionably, the lever throw during the vagus beat was greater than in the other beats recorded by the Hürthle manometer, so that the systolic pressure was actually less than that recorded in the vagus beats. This failure to control the fling of the manometer lever renders these records unsatisfactory evidence.

In the second place satisfactory evidence is not at hand to show that variations in pulse pressure due to heart rate changes do not mask blood pressure changes.

The fact<sup>2</sup> that the lower diastolic pressure and increased pulse pressure accompanying vagus stimulation shown in figure 3 is attended by smaller sphygmomanometer oscillations is seemingly conclusive that the change in diastolic pressure chiefly determines the amplitude in oscillation and that pulse pressure changes play an insignificant part. Another explanation is more plausible, however. With the fall of diastolic pressure the systolic pressure also falls and, to judge from the typical impact waves of the sphygmomanometer record, it falls so low that the extra-arterial pressure is always obliterating the artery and, in consequence, this amplitude is necessarily smaller. During the first vagus stimulation when the diastolic pressure is practically the same, only the first three waves, in which the systolic pressure is somewhat higher, gave anything that might be called a sphygmomanometer record. As soon as extra-arterial pressure is no longer above intra-arterial, as shown in the third vagus stimulation, we get, allowing for friction irregularities, a correspondence between oscillations and pulse pressure changes.

Inasmuch as a clear decision in the matter is imperative to determine the influence of cardiac rhythm on blood pressure changes, it was deemed desirable to reinvestigate these questions.

#### EXPERIMENTS.

The sphygmoscope attachment of Erlanger's blood pressure apparatus was changed to the extent that a Frank's segment capsule, recording by a reflected band of light on a moving bromide film, was substituted for the tambour. By this change the quality of the apparatus was improved to such an extent that now, at least, the variations of pressure transmitted by the elastic ball of the sphyg-

<sup>2</sup> Erlanger, J., and Festerling, E. G., *loc. cit.*, p. 379.

## 6      *Does Cardiac Rhythm Determine Blood Pressure Variations?*

moscope system were accurately picked up by the segment capsule and registered without friction on the writing surface. The sensitiveness was at the same time increased to such an extent that the pressure variations from the dog's femoral could be directly recorded. This was considered a distinct advantage, inasmuch as it is necessary in interpreting pressure measurements in man to deal with the elasticity coefficients of the overlying tissues and of the sphygmoscope system under different tensions and variable air contents. To this end, a conical cuff, containing a trapezoidal bag having a width of seven centimeters, was strapped to the thigh, and by special clamps so fastened to the tightly drawn skin that slipping was not possible. Preliminary tests showed that the pulsations in the artery peripheral to the bag, recorded by an exceedingly sensitive optical arrangement, were entirely prevented when the extra-arterial pressure was five to ten millimeters above the maximal pressure recorded from the other femoral by a maximum mercury manometer. In order to obviate the criticism, which according to the writer's opinion is unjust, that a considerably larger component of the pressure is lost in applying pressure to a flat and conical leg of a dog than by a cuff to the cylindrical arm of man, an arteriograph was applied to the carotid artery in three experiments. The variations in carotid pressure were recorded by Frank's optical manometer,<sup>8</sup> while the maximum and minimum pressure was read as from the other femoral artery. The normal variations in heart rate, the effects of natural breathing, of cutting vagi and stimulating either end, of compressing the trachea, and of atropin and adrenalin as these bore upon the relation between the two curves, were all investigated.

The results of eight such experiments were conclusive. They fail to show that where a method is employed similar to that used in man, variations of diastolic pressure due to changes in heart rate determine variations in the amplitude of oscillations of the sphygmoscope. Specific instances are shown where an extra-arterial pressure not more than ten millimeters below maximal in the other femoral was applied by the bag to the artery. In figure 1 the size of the

<sup>8</sup> In the later experiments the manometer was calibrated.

sphygmoscope oscillations 1 and 2 decreases when systolic pressure of the corresponding beat falls and the diastolic pressure remains unaltered (decreased pulse pressure). In figure 2 a progressive rise of diastolic pressure, due to an accelerated heart, causes not a larger but a smaller oscillation. In this case the highest maximal intra-arterial pressure reached was 167, the minimal was 75, and the extra-arterial was 150.

On the other hand, these records show that when variations in cardiac rhythm occur, an exact correspondence obtains between amplitude of oscillation and pulse pressure. Thus, in figure 1, when, during inspiration, the pulse pressure decreases, diastolic pressure remaining practically unchanged (waves 1 a, 1, and 2), the size of oscillations diminishes and what is of fundamental importance as showing the minimal determining influence of diastolic pressure, this relation held true for the entire range of extra-arterial pressures from above systolic to below diastolic. The three arteriograph experiments were particularly convincing upon this point. Records of experiments of which a few segments are shown in figure 3 illustrate this so clearly as to render detailed discussion superfluous. Experiments taken from the animal's leg with a cuff showed the same relations when the animal was in apnea, but, as the results of table I indicate, the movements incident to respiration in the dog occasionally impair an exact correspondence. The results are appended to show that even when extraneous influence sometimes impairs the results, the relation of the external to the diastolic pressure is always without effect in altering the relation between pulse pressure and sphygmomanometer oscillations. Inasmuch as a decrease in pulse pressure associated with a shorter cycle means a higher diastolic pressure, it follows that, contrary to the interpretation of Erlanger and Festerling, an increase in diastolic pressure in such cases is indicated by the smaller pulse pressure waves. It is necessary to lay stress upon this because the diastolic pressure changes will be misinterpreted when cardiac rhythm is not regular, if the size of the oscillations is taken as the criterion of diastolic pressure. This, of course, does not necessarily imply that larger oscillations may not accompany a rise of diastolic pressure. Figure 1, waves 3 and 4, show that, during natural breathing when

## 8      Does Cardiac Rhythm Determine Blood Pressure Variations?

TABLE I.  
Experiment 13.

Observation.	Maximal intra-arterial pressure.	Extra-arterial pressure.	Minimal intra-arterial pressure.	A. Variation in diastolic pressure (Frank's manometer).						
				B. Variation in pulse pressure (Frank's manometer).			C. Variation in size of oscillations (sphygmomanometer).			
2	126	130	96	+	-	+	+	-	+	+
				-	+	-	-	+	-	+
				-	+	-	-	o	-	+
3	128	152	98	+	-	+	-	+	+	+
				-	+	-	+	-	o	
				+	?	-	+	-	+	
4	118	118	92	-	+	-	-	+	-	+
				+	-	+	o	-	+	-
				+	-	+	-	+	-?	-?
5	118	100	90	-	-	-	+	+	-	-
				+	o	+	-	-	+	+
				+	o	+	-	-	+	+
6	118	90	88	+	-	-	o	+	-	-
				-	+	o	o	-	o	+
				-	+	+	-	o	o	+
7	?	80	88	o	-	+	-	-	-	+
				o	o	-	o	+	o	-
				-	+	-	+	+	+	-
8	138	70	90	-	o	-	-	-	-	+
				+	o	o	o	o	o	-
				?	-	-	+	o	o	-
9	160	85	88	o	-	+	+	-	-	+
				o	+	-	o	+	o	-
				o	+	-	-	+	+	-
10	160	160	88	-	o	+	-	o	-	
				o	o	-	+	o	o	
				o	-	-	+	o	o	

the heart is regular, large oscillations often accompany an increase in diastolic pressure, but the greater coincident pulse pressures may account for the changes. Nor do these experiments necessarily disprove the possibility that the amplitude of oscillation may be determined by the relation of intra-arterial diastolic pressure to extra-arterial pressure when heart rate and pulse pressure remain unaltered. Inasmuch as such conditions are rarely approximated in man, however, they scarcely permit the application of the criterion to establish human pressure variations.

\* + = an increase; — = a decrease over the preceding wave; o = no change.

Since variations in amplitude of oscillations parallel changes in the pulse pressure, it became desirable to investigate whether the troughs and crests of the sphygmoscope oscillations registered by photographic means follow variations in systolic and diastolic pressure of Frank's manometer. If the volume changes of an artery induced by a pressure variation within the vessel could be transmitted to an ideal manometer system externally applied and sustaining a pressure less than systolic, they would induce similar but much smaller changes in this manometer. If these could be recorded without error, they would be proportionate to the variations of intra-arterial pressure. Hürthle (26) attempted to apply this principle in exact estimation of human pressures. Although Frank (27) has pointed out that this can not be realized with such a method, it may still be conceived that an instrument of much lower quality may show a directional but not a quantitative change in pressure.

A comparative study of animal experiments shows that, during apnea, *e. g.*, after curare, during central vagus stimulation, and after adrenalin, an exact qualitative but by no means a quantitative correspondence exists. The troughs and crests, beat by beat, change in direction with the diastolic and systolic pressures of Frank's manometer (figure 2). This correspondence holds not only when the extra-arterial pressure is equal to the diastolic within the artery but is equally faithful up to the systolic level.

A study of the arteriograph records, during which animals were breathing, showed also a general correspondence in the variations of the sphygmoscope record and Frank's manometer (figure 3). Many isolated exceptions were present, however, in the records taken from the leg which it is necessary to analyze in order to recognize the limitations of the method in man. Thus in figure 1, to judge from waves 1 and 2 of the sphygmomanometer record, both systolic and diastolic pressures are falling. A glance at the corresponding pressure waves shows, however, that systolic pressure alone fell in wave 1 and rose again in wave 2. Again, wave 4 gives the impression that an increase in both systolic and diastolic pressure over the preceding beat occurred, whereas the direct pressure record shows that systolic pressure decreased and diastolic pressure remained unchanged.

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An analysis of the cases where discrepancies occurred showed that they could be referred to extraneous movements imparted by respiratory or voluntary muscular contractions of the limb to the bag. The latter are easily recognizable on the curves from animals and man (figure 4), and when present the records may be discarded. The respiratory variations are not always so clearly shown (figure 1), and in the dog they are exceedingly difficult to obviate since every respiration exerts a tug upon the skin of the thigh throughout the inguinal fold of skin connecting the abdomen with the thigh. It follows that the troughs and crests of individual sphygmomanometer variations follow the direction of systolic and diastolic pressures within the artery accurately only when extraneous influence can be guarded against. The experimental plan of the method, therefore, shifts to man.

Fortunately, on account of anatomical differences, these movements can probably be controlled with attention to a proper placement of the arm. If an arm bag is applied to the arm hanging to the side, one may obtain marked variations of the whole curve with respiratory movements which disappear entirely or become altered in direction when the arm is allowed to rest comfortably in a horizontal position away from the chest. In control experiments no variations that could be attributed to such an influence were discernible when a second narrow cuff with a pulse-obliterating pressure was placed above the recording cuff.

It may be concluded then, that, during natural breathing, when precautions are taken that no extraneous movement is transmitted to the cuff, the crests and troughs recorded by an air-tight sphygmoscope system give respectively a directional indication of systolic and diastolic pressures occurring in man.

### THE RELATION BETWEEN SYSTOLIC AND DIASTOLIC PRESSURES AND THE LENGTHS OF THE CARDIAC CYCLES IN MAN.

*Method of Experimentation.*—The subjects reclined upon a bed, their left arms, strapped with a blood pressure cuff, resting away from the thorax. Each subject unacquainted with the objects of the experiment was allowed to rest quietly for several minutes. The systolic pressure was then roughly determined by palpating for

the return of the radial pulsation and the instrument set so that the pressure was 10 to 15 millimeters below this level. The oscillations of the sphygmoscope transmitted by an air-tight system to a Frank's segment capsule, together with the movements of a respiratory tambour communicating with a pneumograph and a tuning fork with a vibrating period of 0.02 of a second, were then projected upon a moving bromide film.

As a rule, the breathing of these quietly resting subjects was entirely involuntary and shallow. When a record had been obtained under these conditions the subject was told to breathe perfectly naturally. The psychic element thus introduced resulted, without fail, in deepening the breathing. This respiration, however, did not exceed in depth that of the normal individual. It is designated as voluntary respiration to distinguish it from the involuntary type naturally assumed. In a number of cases the effects of deep breathing and apnea were also recorded.

*Method of Analyzing the Results.*—The developed records obtained from thirty-five different subjects were analyzed as follows: The cardiac waves were consecutively numbered and their relation to inspiration and expiration was determined. The duration of each cardiac cycle was next expressed in hundredths of a second. By the aid of the millimeter scale photographed on the film, the relative relations of the troughs and crests of the waves were directly determined and expressed in terms of millimeters (figure 5).

These figures, which, according to previous discussion, could give only a criterion of qualitative changes in systolic and diastolic pressures occurring during consecutive beats, were plotted on the ordinates of cross-section paper.<sup>5</sup> To facilitate plotting, the beats were laid off as equal lengths on the abscissæ, the actual duration of the cardiac cycle being numerically indicated for each wave. In this way it was possible to study whether the variations in diastolic and systolic pressures of consecutive waves<sup>6</sup> accorded with those ex-

<sup>5</sup> As the relative changes in systolic and diastolic pressures were alone of importance the actual figures have been omitted from the plot.

<sup>6</sup> The diastolic pressure for any wave (*e. g.*, wave 1, figure 1) is considered throughout the paper as the lowest pressure reached previous to the systole of that beat (point a), and the term systolic pressure is applied to the highest pressure reached during the systole of that beat (point b).

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pected, provided the length of the preceding cardiac cycles solely determined these pressures. In order to do this it is essential to bear constantly in mind the anticipated effects of any change in heart cycle.

It is generally recognized that a lengthening of the cardiac cycle increases, and a shortening decreases the mean pressure of the succeeding beat. This, however, only imperfectly relates the pressure effects, since the inference might be drawn that both systolic and diastolic pressures necessarily follow the directional change of the mean pressure, which is not always the case in animals. When, for example, variations in cardiac rate solely determine the blood pressure changes in anesthetized animals in apnea, as shown by Frank's mirror manometer ( $N = 170$ ), it is the rule that shortening of a cardiac cycle increases the diastolic and decreases the systolic pressure of the beat following (figure 2, *a* and *b*). The same changes occur in naturally breathing animals when cardiac rhythm controls the pressure (figure 7). The systolic pressure deviates from this rule, however, (*a*) when peripheral constriction is great (after adrenalin or asphyxia), (*b*) when the shortening of the cycle is not sufficient to alter the degree of filling (in very slow heart beats), and (*c*) when a progressive acceleration lasts for a considerable period so that the systolic pressure is supported by the diastolic (figure 3, *c*, *d*, *e*). Conversely, a lengthening of the cardiac cycle causes an elevation of the systolic and a fall of the diastolic pressure of the following beat. Here also the systolic pressure may fall under conditions opposite to that outlined above. In all cases, however, the pulse pressure decreases when the heart accelerates, and increases when it slows.

In dogs with rhythmic hearts, or where respiration controls the pressure variations, inspiration causes a fall of both systolic and diastolic pressures, and expiration a rise (figure 1). The pulse pressure increases during expiration and decreases during inspiration. Systolic and diastolic pressures change in the same direction.

*Results.*—To facilitate an analysis the experiments were grouped in accordance with the points they illustrated, and from these groups a number of representative plots are briefly discussed (figure 8).

## INFLUENCE OF HEART CYCLES DURING APNEA.

*Experiment 32.*—Breath held in expiration. Very slight variations (0.01 or of a second) in rhythm occur and the systolic and diastolic pressure of consecutive beats do not vary.

*Experiment 35.*—Voluntary apnea. Heart cycles increase in length slightly (0.02 of a second). This is without influence on systolic pressure, but a slight decrease in diastolic pressure is evident. At the fifth beat, *i. e.*, after a series of progressively lengthening beats, the systolic pressure falls, due to lack of diastolic support.

*Experiment 33.*—Voluntary apnea with very slow heart rate. A lengthening of the third cycle plotted causes a fall of diastolic pressure and a decrease in the systolic pressure of the fourth wave. A shortening of the fourth cycle in the fifth wave increases both diastolic and systolic pressures.

*Experiment 36.*—Voluntary apnea. Average cardiac cycles. A progressive decrease of the cardiac cycles during the first five waves results in a progressive rise of diastolic and a fall of systolic pressures. At the sixth and seventh waves, the systolic pressure rises, due to increased diastolic support resulting from a continued decrease in length of heart cycles. As the cycle increases again in the seventh wave, the diastolic pressure decreases, but systolic does not rise for a beat, probably because the systolic pressure was already high due to diastolic support.

*Experiment 37.*—Voluntary apnea. A progressive and pronounced lengthening of the heart cycle takes place. The progressive decrease of diastolic pressure is accompanied by a systolic rise in the first three waves. The systolic pressure does not continue to rise on account of the greater diastolic drop and, for the same reason, it falls in the sixth and seventh waves.

These experiments, which were the only ones in a series of twenty apnea experiments in which marked cardiac variation occurred during apnea, show that when respiration is in abeyance, variations of cardiac rhythm, except when very small, modify the pressures of man in precisely the same manner as was above stated to be the case in animals.

From this the rule may be formulated that the duration of the preceding cycle probably cannot be considered to govern the pressures unless it can be shown that:

- (1) a decrease in systolic pressure is (*a*) preceded by a cycle shorter than that of the wave before (*e. g.*, waves 4 and 5, experiment 36), and accompanied by an increase of diastolic pressure, or (*b*) preceded by a cycle of the same or a longer length than that of the wave before, and by a decrease of diastolic pressure for several previous beats (*e. g.*, waves 5, 6, and 7, experiment 37); and,
- (2) an increase in systolic pressure is (*a*) preceded by a cycle

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longer than that of the wave before and accompanied by a decreased diastolic pressure (*e. g.*, waves 2 and 3, experiment 37), (*b*) preceded by a cycle of the same or a shorter length than that of the wave before, and by an increase of diastolic pressure for several previous beats (*e. g.*, waves 6 and 7, experiment 36).

### INFLUENCE OF RESPIRATION WITHOUT CHANGES IN CARDIAC RHYTHM.

*Experiment 21.*—Involuntary breathing, moderate depth. After the first wave plotted the cardiac cycles are equal. In spite of this fact systolic pressure falls distinctly during inspiration.

*Experiment 17.*—Involuntary breathing, moderate depth. The duration of the cardiac cycles determining the first five beats is practically uniform. In spite of this the systolic pressure undergoes a fall during inspiration and a rise during expiration which can only be accounted for as an influence of respiratory movement on blood pressure.

*Experiment 24. Curve A.*—Involuntary breathing, moderate depth. Although the heart cycles are equal, systolic pressure is somewhat lower during inspiration and higher during expiration. *Curve B.*—Voluntary, deeper breathing. The inspiratory phase is prolonged and the fall of both systolic and diastolic pressures during this phase is more prominent. Since the cardiac cycles determining waves 2 and 3 and also 4 and 5 are of the same length, the pressure change may be attributed to the effect of inspiration. The pressure changes of wave 6, on the other hand, might be attributed to a cardiac influence.

The experiments, of which these are illustrations, show that, in cases where cardiac variations are negligible or absent, mechanical respiratory influences lower the systolic pressure when breathing is shallow, and the diastolic pressure also when breathing becomes somewhat deeper. As in animals, systolic and diastolic pressures vary in the same direction.

### INFLUENCE OF SLIGHT VARIATIONS IN CARDIAC RHYTHM DURING NATURAL RESPIRATION.

*Experiment 35. Curve A.*—Shallow, involuntary breathing. A fall of systolic pressure during inspiration occurs. Cardiac rhythm is not responsible for the change because the fall of systolic, with a slight tendency to a rise of diastolic pressure, follows a consecutive lengthening of the cardiac cycles; and although a variation in the duration of the cardiac cycle occurs during the two beats of inspiration, the pressure remained unaltered for these beats. *Curve B.*—Deeper voluntary breathing. Waves 2, 3, 4, and 5 might be accounted for by cardiac rhythm, but the systolic decrease of the sixth wave could scarcely be attributed to the lengthened cycle preceding it, nor could the fall of diastolic pressure for the seventh wave be accounted for by the shorter cycle of wave 6.

*Experiment 22. Curve A.*—Shallow, quiet breathing. A very slight reduction of systolic pressure coincident with inspiration occurs. The slight variations

of waves 1, 2, 3, and 4 during expiration are easily explained by heart rate variations. The fall of systolic pressure of wave 5, though slight, cannot have been due to such variation. *Curve B.*—Voluntary breathing. The sudden increase in systolic pressure of waves 3 and 4 could not possibly be attributed to heart rate variations. Nor could the fall of systolic and diastolic pressures during waves 7 and 8 be referred to the shortened cycles preceding them.

*Experiment 19. Curve A.*—Shallow, involuntary breathing. The shortest cardiac cycles do not appear during inspiration and the cardiac cycles determining the first to the fourth waves are practically of the same duration. Still the systolic pressure falls during inspiration. The short cycles occur in expiration where wave 5 is followed by an unexpectedly high systolic pressure with no corresponding increase in diastolic pressure. Upon increasing the depth of respiration (*curve B.*), the systolic and diastolic pressures follow the respiratory phases exactly. The determining cycles for waves 2 and 3 do not vary greatly, yet systolic and diastolic pressures both fall. Waves 4 and 5, determined by the same cycles, show a pronounced difference in pressure not to be accounted for by rate variation.

*Experiment 32.*—Finally, in figure 5, is shown a sample record of the pressure variations obtained during shallow breathing. During inspiration, which begins after the second wave, a fall of systolic pressure takes place. During expiration, which begins after the third beat, the systolic and diastolic pressures both increase. The fall in systolic pressure of the third wave is probably not the effect of a shortening of the second heart cycle, for a similar shortening in expiration does not reduplicate the directional change in the fifth beat. The rise of diastolic pressure of the fourth beat is also difficult to refer back to a lengthened cycle in wave 3.

Although moderate changes in cardiac rhythm may play a part in modifying systolic and diastolic pressures, the influence of respiration is paramount, for, no matter what relation exists between cardiac and respiratory rhythm, the systolic pressure, at least, always falls during inspiration and rises during expiration in these cases.

#### INFLUENCE OF THE AVERAGE RHYTHM VARIATION (MODERATE) DURING NATURAL RESPIRATION.

*Experiment 25.*—Quiet, involuntary breathing (figure 6). The systolic and diastolic pressures of waves 2 to 5 may be explained as due to cardiac variation, although it cannot be denied that inspiration may play a part in determining waves 4 and 5. Wave 6, following a shorter cycle, shows a higher systolic pressure without previous elevation of the diastolic to account for the systolic rise on a summation basis. On the other hand, it is significant that the deviation occurs at the beginning of expiration.

*Experiment 34.*—Quiet, involuntary breathing. The systolic pressures of waves 2 and 3 during inspiration are equal yet determined by different length cycles. The cycles governing waves 4 and 5 in expiration are equal, but both systolic and diastolic pressures increase in the latter beat.

*Experiment 30. Curve A.*—Quiet, involuntary breathing. Every pressure

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change can be explained by a variation of cardiac rhythm. That respiration has a modifying influence, however, is suggested by the fact that, although the systolic and diastolic pressures of waves 5 and 6 both rise, the pulse pressure is greater. *Curve B.*—When breathing becomes deeper, respiration alters the direction of the pressures, as determined by heart rate variation. The lower systolic pressure of waves 4 and 5 and the higher systolic pressure of wave 6 are directly opposite to those expected if determined by previous heart cycles. The diastolic pressure also fails to agree with heart rate variation.

These cases show that when a considerable variation in cardiac rhythm occurs, especially when cardiac acceleration is limited to inspiration, a casual glance leaves the impression that systolic and diastolic pressures are entirely governed by the previous cardiac cycle. A careful perusal, however, shows that in each case one or more beats occur during every shallow respiration that cannot be accounted for upon this basis, and, in many cases, when breathing becomes deeper the effect of respiration becomes dominant.

### MARKED RESPIRATORY ARHYTHMIA.

*Experiment 29 A.*—Moderately deep, involuntary respiration. Variations in cardiac cycle (0.23 of a second) entirely accounted for many waves not plotted. Wave 2, during inspiration, is governed by the same cycle as wave 1 in expiration, but the latter has a much reduced systolic pressure. The reduced systolic and increased diastolic pressure of the other waves in inspiration may, however, be accounted for by cardiac variations. Waves 5, 6, and 7, having practically cycles of the same length, show an increase of both systolic and diastolic pressure during expiration. It is interesting to note that wave 2, governed by a heart cycle of 0.83 in inspiration, is no larger than wave 7 governed by a cycle of 0.61 in subsequent expiration.

*Experiment 16.*—Moderately deep, voluntary breathing, cardiac cycle variation 0.23 of a second. With a progressive decrease of the cardiac cycle we find a decrease in systolic and an increase in diastolic pressure. The low diastolic pressure of wave 6 and the higher systolic pressure of wave 7 are difficult to accredit to the shorter cycles preceding.

*Experiment 13.*—Involuntary, moderate breathing, cardiac cycle variation 0.30 of a second. The variations of both systolic and diastolic pressures correspond to cardiac variations entirely. Wave 3, following a longer cycle, shows the characteristic lower systolic pressure which is the rule in slow hearts. A respiratory influence may exist but its invocation to explain the changes is not necessary.

*Experiment 7.*—Moderately deep respiration, cardiac variation 0.29 of a second. Systolic and diastolic pressure can be accounted for by heart rate variation. The rise of diastolic and fall of systolic with consecutive shortening until after the fifth wave leads to an increase of systolic pressure due to summation, is typical of cardiac rate changes. No necessity exists for the assumption of intervention by respiration.

*Experiment 18.*—Dog trained for calorimeter work. Figure 7 shows the

characteristic pressure variations of the normal, unanesthetized dog. Such pronounced variations in rhythm are never encountered in man. With inspiratory acceleration the diastolic pressure rises and systolic pressure falls until after several beats the summated effect causes the systolic pressure also to rise slightly. No evidence is found that mechanical movements of respiration play a part.

It may be concluded that in cases where extreme variations in cardiac rhythm occur these variations often obscure entirely any effect that movements of respiration may have on the systolic and diastolic pressures.

#### CONCLUSIONS.

Changes in cardiac rhythm are not the only determinants of blood pressure variations in man. They play a part in the variations of systolic and diastolic pressures, the relative importance of which depends on the degree of arrhythmia present and on the depth of respiration which, in itself, causes the systolic and diastolic pressures to decrease during inspiration and increase during expiration. No degree of rate variation can be regarded as a type. The cases range from those in which respiration governs the change of pressures entirely through those where more or less complicated mixtures of heart rate and respiratory influences intermingle to those in which extreme cardiac variations alone determine the pressure changes.

The emphatic insistence of Henderson and his collaborators that heart rate changes play a more important part than is commonly recognized in man is true in many cases. That, in certain cases, it is the only determining influence may also be admitted, but they are in the minority. The majority show the intervention of a respiratory influence which controls, at least, the variations of systolic pressure. In the light of these results, the doctrine that, in man, an effective venous pressure exists sufficient during all respiratory phases to insure superimposable beats, must be subjected to further reinvestigation.<sup>7</sup>

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### EXPLANATION OF PLATES.

#### PLATE I.

FIG. 1. Natural respiration. The amplitude of the sphygmoscope oscillations corresponds to pulse pressure changes and not to variations in diastolic pressure. Troughs and crests fail to follow intra-arterial systolic and diastolic pressures if breathing movements are communicated to limb. R = respiration, inspiration up-stroke; P = carotid pressure recorded by Frank's optical manometer; S = sphygmoscope oscillation. The numbers are referred to in the text. About three fourths actual size.

FIG. 2. Apnea. Close correspondence of troughs and crests of sphygmoscope record with systolic and diastolic changes recorded by Frank's manometer. The lettering is the same as in figure 1. About two thirds actual size.

#### PLATE 2.

FIG. 3. Five segments of records, showing that variations of pulse pressure occasioned by rhythmic cardiac changes determine the amplitude of the sphygmoscope oscillations obtained from an arteriograph. In the upper curve alone, where the intra-arterial systolic pressure fell below extra-arterial, and conse-

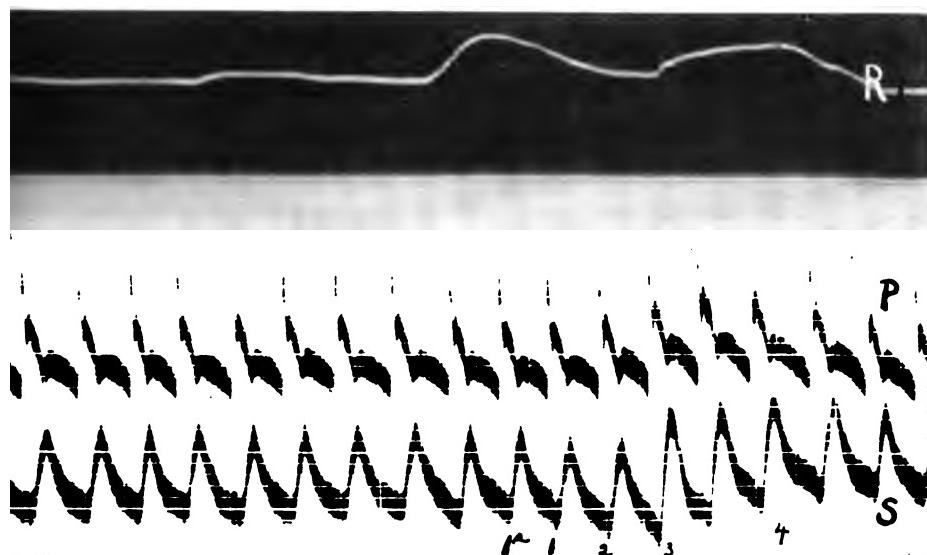


FIG. 1.

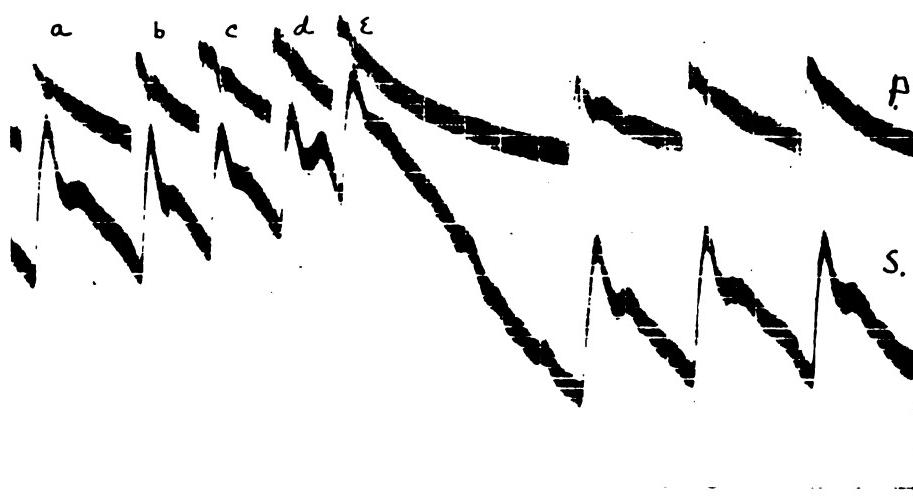


FIG. 2.

(Wiggers: Cardiac Rhythm.)



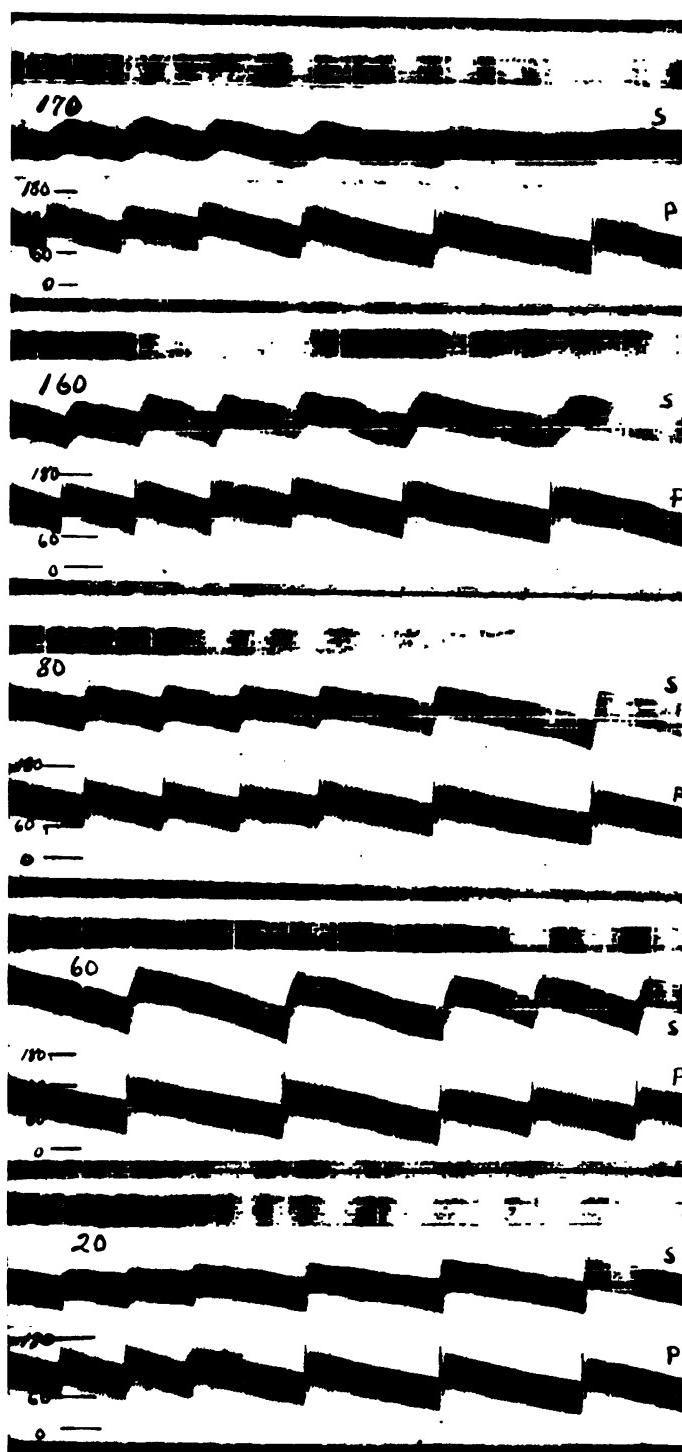


FIG. 3.

(Wiggers: Cardiac Rhythm.)



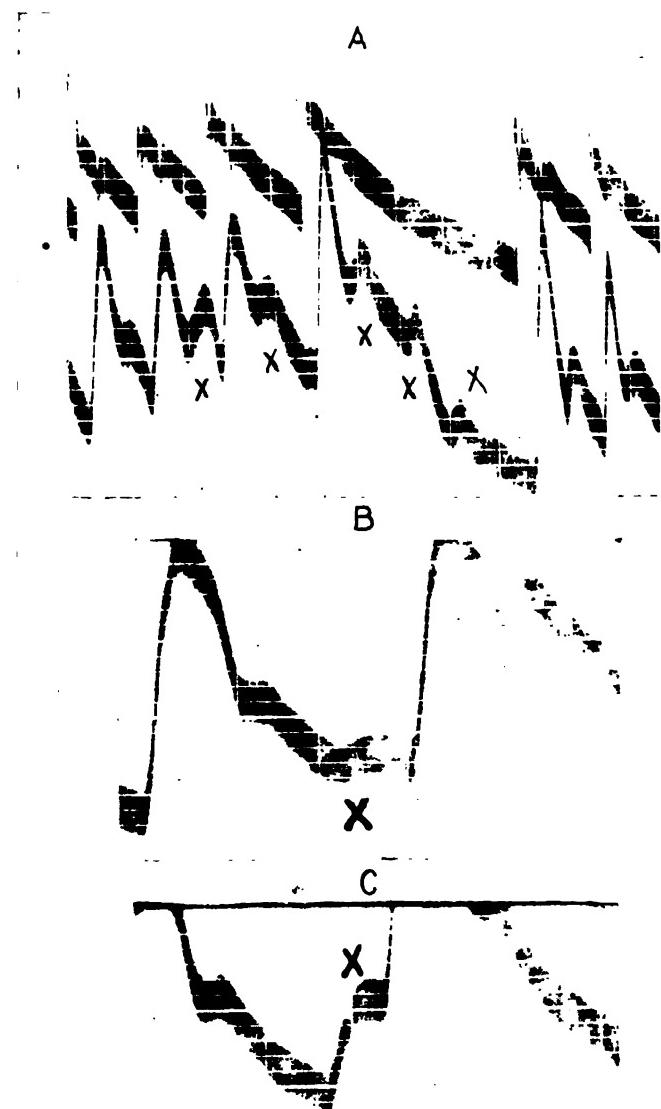
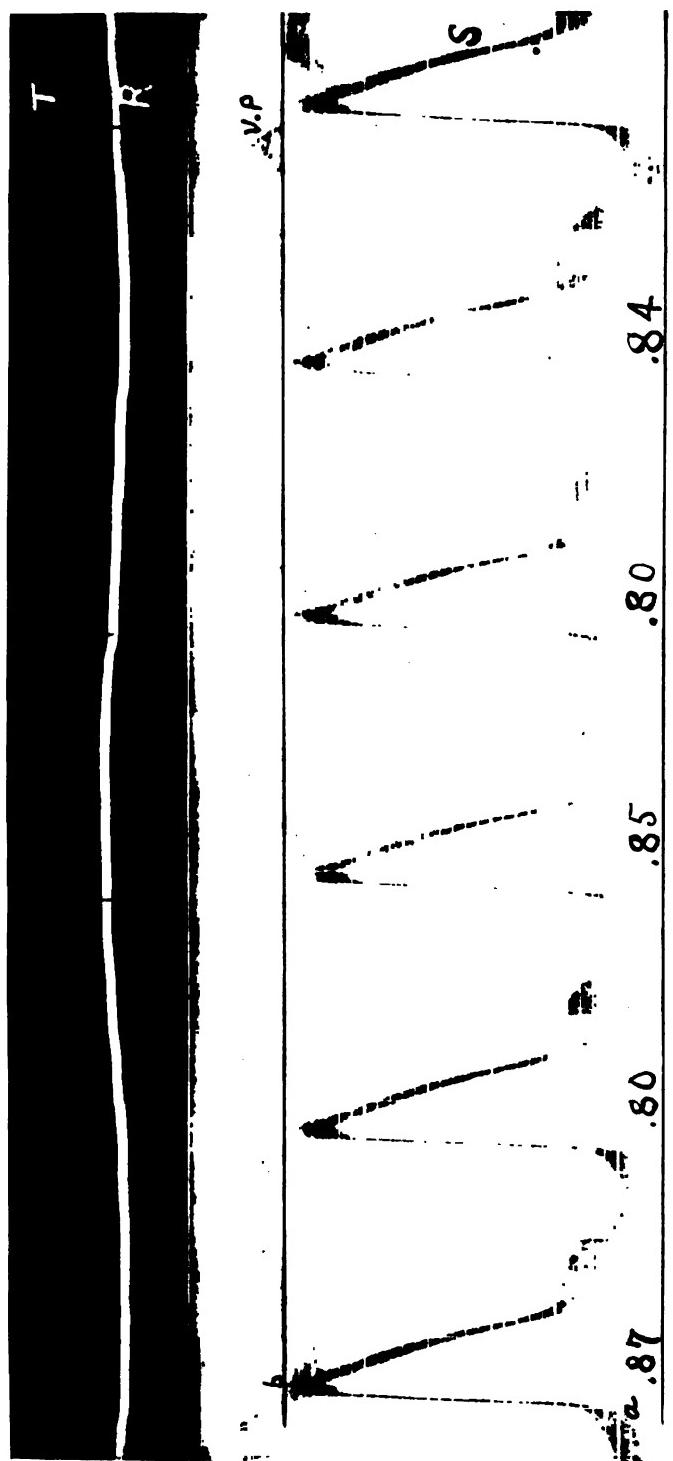


FIG. 4.

(Wiggers: Cardiac Rhythm.)





(Wiggers: Cardiac Rhythm.)

FIG. 5.



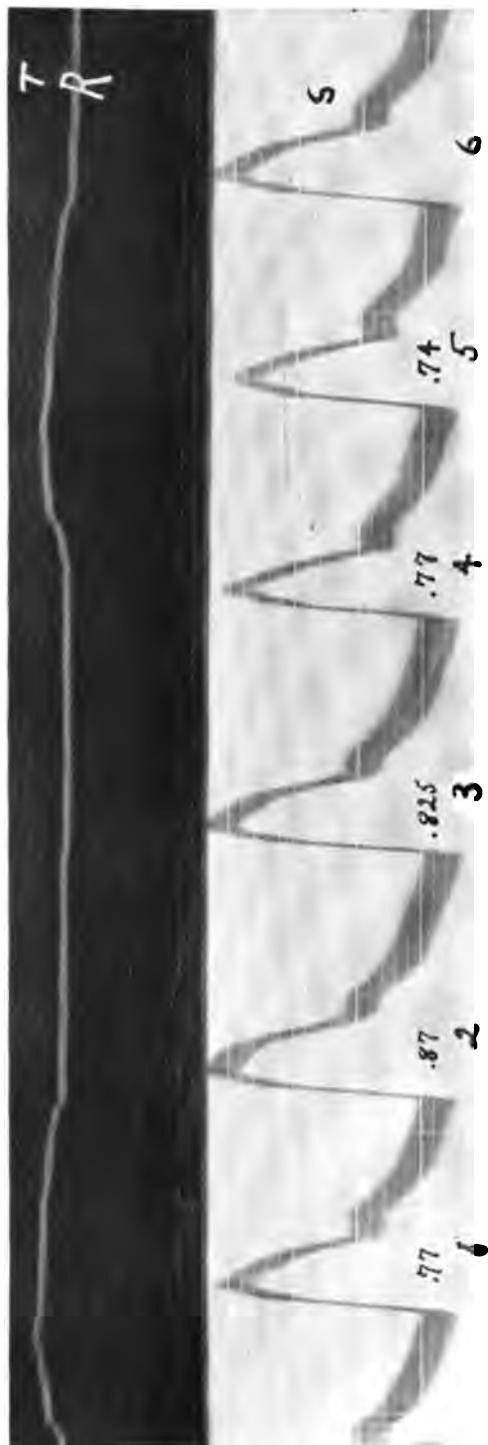


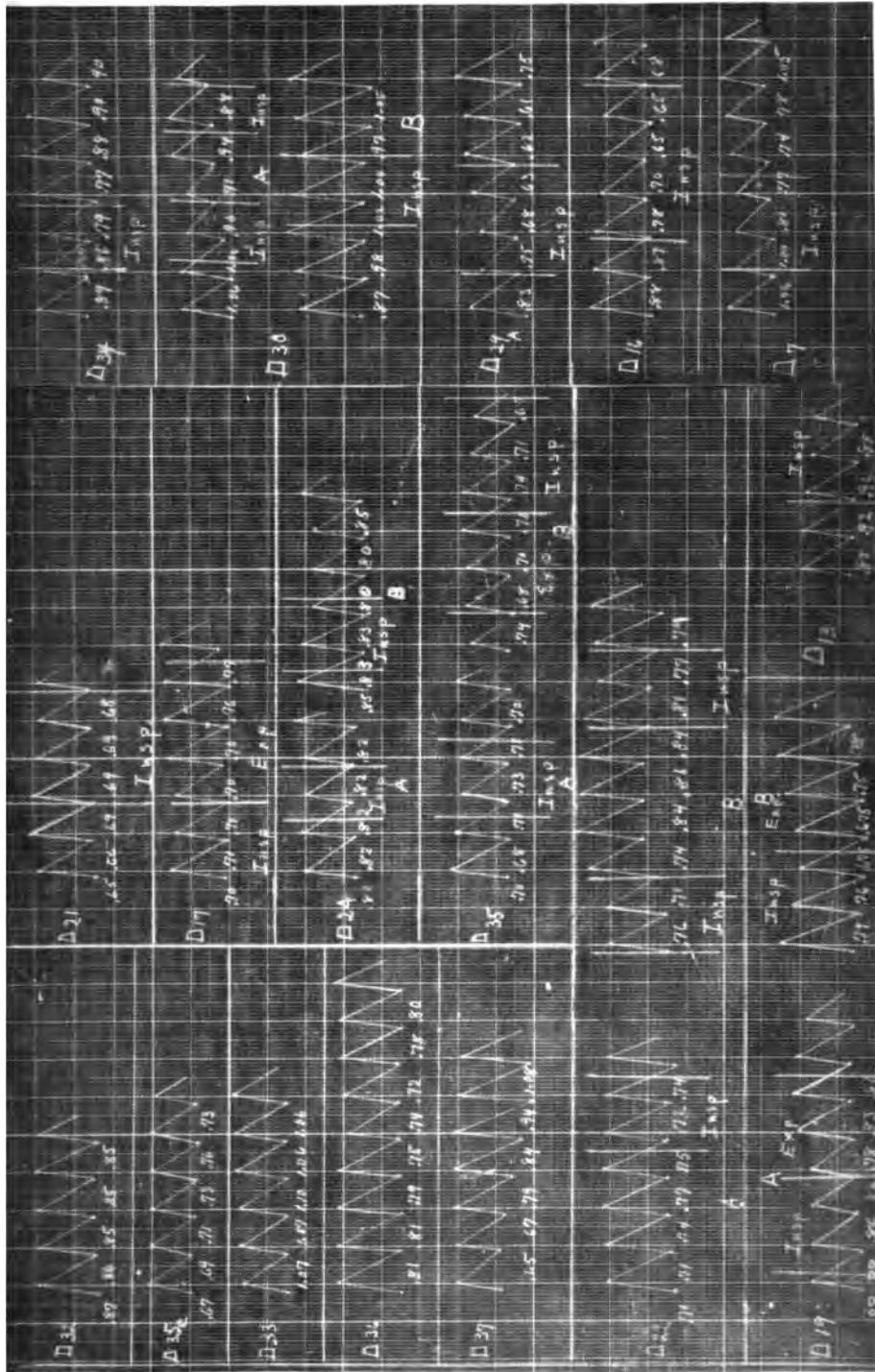
FIG. 6.

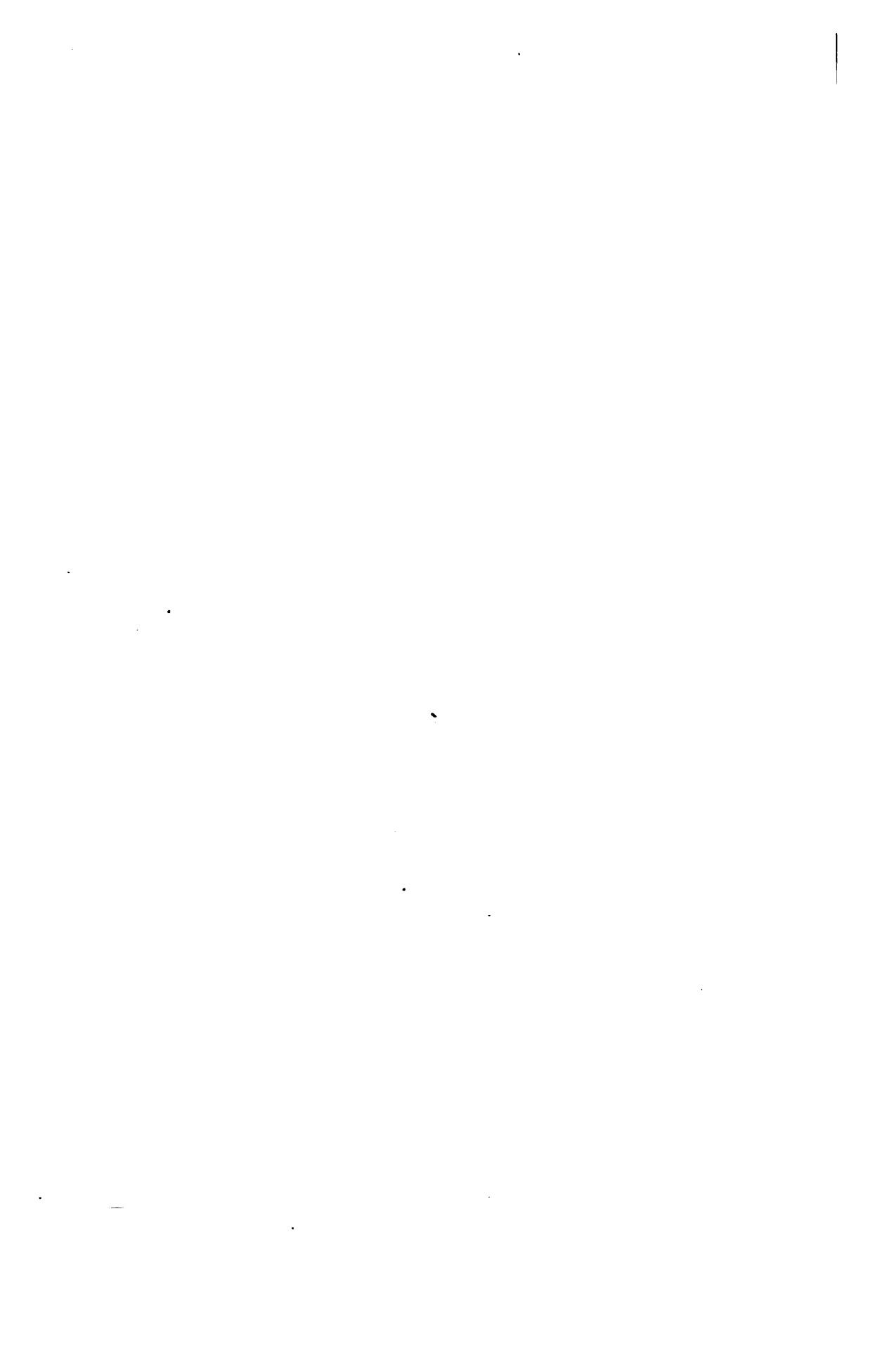


FIG. 7.

(Wiggers: Cardiac Rhythm.)



Fig. 8.  
Wiggers: Cardiac Rhythms.)



quently obliterated the vessel, does a fall of diastolic pressure together with an increase of pulse pressure result in smaller oscillations. The lettering is the same as above. B = the base line from the top of which calibration was made, hence the calibration scale on Frank's pressure record (P) applies to the top of the line. About two thirds actual size.

PLATE 3.

FIG. 4. Three segments of records showing at X the effect of extraneous muscle contractions, (A) in the dog, and (B and C) in man. Actual size.

PLATE 4.

FIG. 5. Human pressure variations, involuntary shallow breathing, slight heart rate changes. T = time in 0.02 of a second. R = respiration, inspiration upstroke. S = sphygmoscope oscillations. V. P. = jugular pulse, of no significance in present connection. About five sixths actual size.

PLATE 5.

FIG. 6. Human pressure variations, involving quiet breathing. Average rhythm variation. The lettering is the same as before. About five sixths actual size.

FIG. 7. Pressure variations of an unanesthetized dog resting and breathing quietly. Marked cardiac arrhythmia. About actual size.

PLATE 6.

FIG. 8. Plots from experiments showing the relation of systolic and diastolic pressures to heart cycles and respiration.

## OBSERVATIONS ON THE GROWTH OF BACTERIA ON MEDIA CONTAINING VARIOUS ANILIN DYES.\*

By CHARLES KRUMWIEDE, JR., M.D., AND JOSEPHINE S. PRATT.  
*(From the Research Laboratory of the Department of Health, New York.)*

In recent communications Churchman (1) has described a selective action on bacteria of gentian violet. Stated in general terms, he found that bacteria were divided into two groups, of which one fails to develop in media containing gentian violet, while the other grows abundantly in the presence of this dye. The former group corresponds roughly with the Gram-positive group, the latter with the Gram-negative group of bacteria. His conclusions are based mainly on results obtained with dilutions of 1 to 100,000 of the dye.

A striking variation was noted in some enteriditis strains. These strains were identical by all ordinary cultural tests; one strain, however, persistently refused to grow on gentian violet agar. A subsequent examination showed that this strain could be differentiated from the others by agglutination.

Our interest in the use of media containing dye stuffs was further aroused by an article by Signorelli (2). He stated that on agar containing dahlia, cholera vibrios develop colored colonies by absorption of the dye with decolorization of the medium, whereas non-cholera vibrios give colorless colonies. We (3) were unable to verify these findings. In the course of the work we found two non-cholera vibrios which uniformly refused to grow on agar containing dilutions of dahlia which had no influence whatever on the growth of similar vibrios. Because of the above results, a few preliminary observations with various anilin dyes were made, which showed differences among related bacteria, and also differences in the reaction of the same bacteria to different dilutions of the same dye. The results of the comparison of the action of various dyes are given in this paper.

For the tests, the common pathogenic bacteria which grow freely

\* Received for publication, September 2, 1913.

on ordinary agar were selected. *Bacillus subtilis* was included as it is very sensitive to the presence of gentian violet. Other bacteria requiring serum or other similar additions were tried in a limited way, but this was discontinued as the albumins interfered with the activity of the dye.

As a routine a batch of agar was selected which was found especially suitable for the more feebly growing types. To the hot agar an appropriate volume of a watery solution of dye was added to give the final dilution desired. The same agar was used in each experiment.

The most convenient and economical method was found to be as follows. One unopened Petri dish was used to tilt up one side of a second dish. In the opposite side was poured just sufficient agar to give a satisfactory slant. This dish was covered and used to tilt up a third dish, and so on in a row. After the agar had set, slants were poured in the other side of the Petri dishes which were tilted in the reverse direction. In this way two mixtures of agar could be used in the same dish, very little agar being required for each slant.

In the general observations that follow two dishes were used containing the control agar, and agar with 1 to 500,000, 1 to 100,000, and 1 to 50,000 dilutions of the dye. The weakest dilution was used in the plate with the control.

For inoculation a fresh broth culture was usually employed. A small loop was used and a streak made on the agar slant, a fresh loopful being taken for each slant. Very heavy seeding as from agar slants tends to give irregular results, probably because of lack of close contact between the bacteria and dyed agar.

Table I gives the results of a series of tests after eighteen to twenty-four hours' incubation.

Some allied bacteria showed variations as noted in the table; namely, the streptococcus-pneumococcus group, the dysentery group, the capsulatus group, and diphtheria and the morphologically allied types. More complete tests of these groups were made.

Naphthylamin blue R gave an apparent difference between diphtheria and diphtheroid bacilli. Thirty-four strains of these bacilli were, therefore, planted on agar containing this dye. There was

*Effect of Anilin Dyes on the Growth of Bacteria.*

TABLE I.

Type.	Bacteria tested.	Hoffman violet..	Crystal violet.	Dahlia.	Fuchsin.	Rosaniline violet.	Cresylichloro-violet.	Anilin violet. Gentian B.	Naphthamin blue R.	Bleu de Lyon.	Säure-violet (Kühne).	Methyl-violet 6 B.
Gram-positive	Staphylococcus aureus.....	-	-	-	-	-	-	-	-	-	-	-
	Streptococcus (green).....	-	-	-	-	-	-	-	-	-	-	-
	Streptococcus (hemolytic).....	-	-	-	-	-	-	-	-	-	-	-
	Streptococcus mucosus capsulatus.....	-	-	-	-	-	-	-	-	-	-	-
	Pneumococcus.....	-	-	-	-	-	-	-	-	-	-	-
	B. diphteriae.....	-	-	-	-	-	-	-	-	-	-	-
	Diphtheroid bacilli (Cameron).....	-	-	-	-	-	-	-	-	-	-	-
	Diphtheroid bacilli (Cunningham).....	-	-	-	-	-	-	-	-	-	-	-
	B. hoffmanni.....	-	-	-	-	-	-	-	-	-	-	-
	B. subtilis.....	-	-	-	-	-	-	-	-	-	-	-
	B. lactimorbi.....	-	-	-	-	-	-	-	-	-	-	-
	B. anthracis.....	-	-	-	-	-	-	-	-	-	-	-
Cholera.....	Vibrio (indol-positive)	-	-	-	-	-	-	-	-	-	-	-
	Vibrio (indol-negative).....	-	-	-	-	-	-	-	-	-	-	-
	B. proteus.....	-	-	-	-	-	-	-	-	-	-	-
	B. pyocyanus.....	-	-	-	-	-	-	-	-	-	-	-
	B. dysenteriae (Shiga).....	-	-	-	-	-	-	-	-	-	-	-
	B. dysenteriae (Flexner).....	-	-	-	-	-	-	-	-	-	-	-
	B. dysenteriae (Mt. Desert).....	-	-	-	-	-	-	-	-	-	-	-
	B. typhosus.....	-	-	-	-	-	-	-	-	-	-	-
	B. paratyphosus, A.....	-	-	-	-	-	-	-	-	-	-	-
	B. paratyphosus, B.....	-	-	-	-	-	-	-	-	-	-	-
	B. enteriditis (Gaertner).....	-	-	-	-	-	-	-	-	-	-	-
	B. coli communis.....	-	-	-	-	-	-	-	-	-	-	-
	B. coli communior.....	-	-	-	-	-	-	-	-	-	-	-
	Friedländer's bacillus.....	-	-	-	-	-	-	-	-	-	-	-
	B. lactic aerogenes.....	-	-	-	-	-	-	-	-	-	-	-
	B. ozanic.....	-	-	-	-	-	-	-	-	-	-	-
	B. pullorum.....	-	-	-	-	-	-	-	-	-	-	-

+ = growth like control; + = restrained growth; x = markedly restrained growth; - = no growth; \* = some growth.

irregular restraint at 1 to 100,000, more marked or complete in some instances at 1 to 50,000. There was, however, no evidence of a grouping according to the different types of bacilli.

On *cresylechtviolett* the strain of pneumococcus failed to grow whereas other allied cocci grew. Forty strains of this group were tested. A variable restraint or inhibition of individual cultures was noted, but this had no relation to the subgroups.

In the dysentery group there is a general tendency for the para-dysentery types to show less restraint than the Shiga type. The result on fuchsin seemed to show a definite difference between the types. The inhibition caused by fuchsin was also of interest because of its presence in Endo agar. This is the most commonly used medium for the isolation of dysentery and must, therefore, have been found satisfactory for this group; at least we know of no unfavorable criticism of the growth on this medium. The question arises as to how far the influence of the dye itself might be lost after its reduction by sodium sulphite to the leuko-base.

Table II gives the results on fuchsin agar with the available strains of dysentery and paradysentery. The table gives the range of growth observed in several repetitions.

It is probable that some of the cultures under similar names, but obtained from different institutions,<sup>1</sup> are duplicates. As we were unable to determine this positively they are given separately in the table. They act as probable controls on the degree of variation of growth in the presence of the dye.

It is evident from table II that the reaction to fuchsin is not a completely specific difference between the dysentery and paradysentery groups. There is a shading of one group into the other. There is, however, a marked tendency to a general group reaction.

Two strains, one of the Shiga type and the other of the para-dysentery type, which reacted almost alike were plated and ten fishings of each made in broth. These were planted on fuchsin agar to determine the probable range of variation under identical conditions. A moderate variation was noticed in some instances slightly greater than those already noted in the table.

<sup>1</sup> We are indebted to the Museum of Natural History, to The Rockefeller Institute for Medical Research, and to Professor Hiss and Dr. Torrey for these cultures.

*Effect of Anilin Dyes on the Growth of Bacteria.*

TABLE II.  
*Growth of Dysentery Bacilli on Fuchsin Agar.*

Culture.	Mannite-fermentation.	Time of incubation and dilution of dye.											
		18 to 24 hours.				48 hours.				72 hours.			
		25,000	50,000	100,000	150,000	25,000	50,000	100,000	150,000	25,000	50,000	100,000	150,000
Shiga, Gay-Shiga, Shiga.....	-	-	-	- or X	-	-	-	s.c. or X	±	-	-	s.c. ±	±
Kruse, Kruse.....	-	-	-	- or s.c.	- or X	-	-	s.c. or X	±	-	-	s.c. or X	±
Kruse, Shiga.....	-	-	-	- or X	X	-	-	X or ±	±	-	-	X	+
Shiga.....	-	-	-	- or X	±	-	-	or s.c.	±	-	-	±	++
Torrey 21.....	-	-	-	X or ±	±	-	-	X or ±	+	+	- or I.C.	X or ±	++
Cruz I .....	-	-	- or X	± or +	+	- or X	±	+	+	- or X	±	+	++
Sydenham.....	+	-	- or X	± or +	+	-	X or ±	+	+	-	X or ±	±	++
Torrey 4, Torrey 5-6, Hiss V.....	+	-	X	± or +	+	s.c. or X	± or +	+	+	-	X or ±	±	++
Hiss V, Flexner-Harris, Medburg, Ferra-Hiss, Torrey 16-3, Torrey 32, V, Baltimore, Strong, Flexner, Mt. Desert.....	+	X	± or +	± or +	+	X or ±	± or +	+	+	+	± or +	+	+
Strong, Gay-Flexner, Silk, Baltimore, Flexner, Gray, Torrey 3, Torrey 41, Flexner-Harris.....	+	± or +	+	+	+	± or +	+	+	+	+	± or +	+	+
Gray B, Hiss 48.....	+	+	+	+	+	+	+	+	+	+	+	+	+

- = no growth; I.C. = colony; s.c. = several colonies; X = very slight growth; ± = evident restraint; + = practically like control.

*Influence of Decolorization on Fuchsin.*—An agar was made containing decolorized fuchsin diluted 1 to 25,000. The strains showing no growth in this concentration of the unaltered dye after twenty-four hours' incubation were planted. Three strains grew as well as on the control, four strains showed slight to moderate restraint, and one strain showed marked restraint. With a similar medium containing the decolorized dye in a dilution of 1 to 50,000, one strain showed some restraint, but at 1 to 100,000 no restraint was evident with any of the strains.

Evidently decolorization robs the dye of some of its action. A series of plates was made containing the decolorized dye in the 1 to 50,000 dilution. These were kept at room temperature and in the light to bring back the color gradually. Several of these plates, with controls, were planted each day. When planted the day after making, some restraint was evident. Those planted thereafter gave no growth except with a strain which grew in the presence of undecolorized fuchsin. The reaction of three members of the dysentery group to the violet dyes has been given. Because of the results on fuchsin the reaction of all the strains was tried, gentian violet and dahlia being used. With both dyes, individual strains showed more variation than with the fuchsin. There was no apparent difference between the dysentery and paradyssentery group, the individual strains of both groups showing, however, markedly different degrees of restraint in the presence of the dye.

*Reaction of the Streptococcus-Pneumococcus Group to Dyes.*—In the results already given there is a marked quantitative difference in the reaction of the streptococcus-pneumococcus group as compared with the other Gram-positive bacteria. Because of this about a hundred strains of the group were tried;<sup>2</sup> these included typical pneumococci, streptococci, *Streptococcus mucosus*, types from chronic endocarditis, streptococci from various diseased conditions in man, a series isolated from the conjunctiva, a series isolated from domestic animals, and a few from the external world. Two dyes were used, gentian violet and Hoffman violet. The results were practically the same with both dyes.

<sup>2</sup>We are indebted to Miss Jean Broadhurst for most of the streptococcus cultures.

In dilutions of 1 to 500,000 of the dye the members of the group grew as freely as on the control, with a few exceptions where slight restraint or delayed growth was evident. By increasing the concentration of the dye, the amount of restraint becomes greater, and some of the strains fail to grow, the number increasing with the concentration. When a dilution of 1 to 100,000 is reached a few strains still grow but the growth is restrained or delayed. One exception was a strain of *Streptococcus lacticus*, which even at 1 to 600,000 showed inhibited or delayed and feeble growth.

The Gram-positive organisms used for comparison, including sixteen strains of staphylococci, failed to grow at 1 to 500,000 dilution of the dye. In a few instances a slight growth developed along the edge of the slant, probably due to the dilution of the dye by the water of condensation.

The streptococcus-pneumococcus group, even the feebly growing strains, is, therefore, more resistant to the action of the violet dyes than other Gram-positive bacteria, growing at dilutions which still inhibit the other Gram-positive bacteria. This may be found of value in isolation from mixed material.

The above observations explain the irregular results in the streptococcus-pneumococcus group given in Churchman's protocols. Stowell, Hilliard, and Schlesinger (4) found regular inhibition of nearly all their strains.

The influence of decolorization by sodium sulphite on the restraining action of gentian violet was very slight. In a concentration of 1 to 50,000 or 1 to 100,000 of the dye, there was no growth of either the staphylococcus or hay bacillus; when a dilution of 1 to 400,000 was reached, there was very feeble growth of the staphylococcus, but not of the hay bacillus.

While the above work was in progress, a further article by Churchman appeared (5). He found similar results, using allied dyes. As he used only four strains for testing, and, as far as we can determine, only one dilution of the dye, he naturally found none of the variations in individual dyes, in which we were especially interested.

## SUMMARY.

Gentian violet and allied anilin dyes have a similar influence on bacterial growth, dividing bacteria into two groups corresponding in general to their reaction to the Gram stain.

Among Gram-negative bacteria a strain is occasionally encountered which will not grow on violet agar, differentiating it from other members of the same species or variety.

The reaction is quantitative, although the quantitative character is more marked with some species than with others.

The streptococcus-pneumococcus group differ from other Gram-positive bacteria in their ability to grow in the presence of amounts of dye sufficient to inhibit the other species.

The dysentery bacillus group shows marked variation in the presence of dyes. In the case of fuchsin the variation approaches closely a specific difference between the dysentery and paradysentery groups. The variations of the latter groups with other dyes show no correlation with the common differential characteristics. A closer study might reveal variations in other characteristics which would parallel the different reactions to dyes. Decolorization with sodium sulphite robs the dyes of some of their inhibitive powers.

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## THE ACID AGGLUTINATION OF PNEUMOCOCCI.\*

By L. J. GILLESPIE, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The method of agglutination of bacteria by acid was introduced by Michaelis (1) for the differentiation of bacterial species. By means of it closely allied types can be distinguished, since the hydrogen ion concentration at which agglutination is maximal is characteristic for the various species.

The determination of the point of maximal agglutination has been compared to the determination of the melting point of a chemical compound. However, it is not possible to determine accurately the point of maximum agglutination for all cultures. On the one hand, no agglutination at any hydrogen ion concentration may occur. This has been found by Beniasch (2) to be the case for *Bacillus coli*, and, indeed, certain strains of nearly all species of bacteria have been found by Beniasch to be non-agglutinable within the tested reaction limits. On the other hand, agglutination may occur in uniform degree over a somewhat broad range of hydrogen ion concentration.

The result of much work on the acid agglutination of the typhoid-colon group of bacilli has shown that *Bacillus typhosus* and *Bacillus paratyphosus* are easily distinguished by means of the reaction. Differences between the paratyphoid bacilli A and B, or C, have not been seen by most workers (Beniasch (2), Jaffé (3), and Heimann (4)).

The writer wished to learn whether certain types of pneumococci (described by Dochez and Gillespie (5)), which are distinguished sharply by means of serum reactions, but incapable of being distinguished morphologically or by cultural tests, can be distinguished and classified by means of acid agglutination.

### THE METHOD OF ACID AGGLUTINATION.

To different samples of bacterial emulsion in distilled water, different hydrogen ion concentrations are imparted, usually varying in

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geometrical progression. The emulsions are incubated at body temperature. Agglutination should occur in some mixtures, and if an optimum is to be recorded, some mixtures should prove to be too acid and others not sufficiently acid for the occurrence of the phenomenon.

The reactions of the bacterial suspensions are controlled by adding to them solutions of a weak acid, or of an acid still further weakened by the presence of one of its salts. By this means low hydrogen ion concentrations are given by relatively high concentrations of acid, so that loss of small quantities of acid in neutralizing alkaline substances from the culture medium is unimportant. In either case the acid concentration is varied in the series of bacterial emulsions; if salt is added the same concentration must obtain in all members of the series. The two equations used for calculating the hydrogen ion concentrations differ only because they are approximations, as the derivation shows.

Applying the mass action law to the electrolytic dissociation of the monobasic acid whose constant is  $k$ , we have,

$(H') \cdot (R^-) = k(HR)$  where  $(HR)$  indicates the concentration of the acid, in gram molecules per liter,  $(H')$  the concentration of hydrogen ions, and  $(R^-)$  the concentration of the  $R$  ions.  $(R)$  includes all  $R$  ions from whatever source. If we increase the concentration of  $R$  ions in a solution of the pure acid  $HR$  by adding a salt  $NaR$ , then we have a molecule of  $R^-$  for every molecule of salt introduced, since the salt is almost completely dissociated; and though the dissociation of the acid retrogresses we must have a molecule of  $R^-$  for every molecule of  $H'$  split off from the acid. We have, therefore,

$$\begin{aligned} (H') \cdot (\text{salt} + H') &= k(HR) \quad (\text{where salt} = \text{the number of molecules of salt introduced per liter}) \\ &= k(\text{acid}) \quad (\text{where acid} = \text{the number of molecules of acid introduced}), \end{aligned}$$

because the acid is mostly in the undissociated state. Therefore

$$(H') = k(\text{acid}) / (\text{salt} + H').$$

Now if no salt is present this reduces at once to

$$(H') = \sqrt{k(\text{acid})}, \quad (1)$$

whereas if salt is added even in moderate amounts,  $(H')$  is then small in comparison with  $(\text{salt})$ , and the equation becomes

$$(H') = k \text{ (acid)}/(\text{salt}). \quad (2)$$

Equation (2) shows that with this type of regulator dilution does not change, within limits, the hydrogen ion concentration. The  $k$  for acetic acid is  $1.8 \cdot 10^{-5}$ ; for lactic acid it is  $1.38 \cdot 10^{-4}$ . The hydrogen ion concentration is given in grams per liter.

#### TECHNIQUE.

All the organisms studied had recently been isolated from patients having lobar pneumonia.

The broth used for the tests was a beef infusion containing 0.5 per cent. of sodium chloride and 1 per cent. of Witte's pepton (Kahlbaum), sterilized intermittently in streaming steam for short periods. The reaction was + 0.6 per cent. acid to phenolphthalein.

The lactate mixtures were prepared from stock solutions of one third normal sodium lactate, with a small crystal of thymol, and normal lactic acid, according to the scheme which follows. They were employed fresh.

N/3 sodium lactate in c.c.	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
N lactic acid in c.c. ....	0.12 <sup>1</sup>	0.25	0.5	1.0	2.0	0.5	1.0	2.0	4.0	8.0	16.0	
	8	8	8	8	8	8	8	8	8	8	8	
Distilled water in c.c. ....	18.4	18.2	18.0	17.5	16.5	18.0	17.5	16.5	14.5	10.5	2.5	
Ratio of acid to salt ...	—	—	—	—	—	—	—	—	—	—	—	
	1	1	1	1	1	1	2	4	8	16	32	
H ion concentration in grams per liter, multiplied by $10^4$ .....	32	16	8	4	2	1	2	4	8	16	32	
	0.04	0.1	0.2	0.4	0.7	1.4	2.8	5.5	11	22	44	

A series of dilute lactates (1 to 6) was also used. It was prepared by adding five volumes of distilled water to one volume of each of the lactate mixtures described above.

Normal acetic acid, when diluted (1 to 1.3) by bacterial emulsion in the manner to be described, gives a reaction somewhat less acid than the 32:1 lactate mixture. It was used, and its reaction was recorded as if it were exactly equivalent to that lactate mixture. By diluting normal acetic acid 1 to 2.86 a reaction mixture can be obtained which when diluted by the bacterial emulsion corresponds exactly to the 16 to 1 lactate mixture. By making progressive dilutions from this N/2.86 acetic acid, diluting 1 to 4 in each case, a series of mixtures was obtained corresponding, when bacterial emulsion is added, to

<sup>1</sup> Read 0.12 c.c. of normal acid freshly diluted 1:8.

the lactate mixtures. In all, six different strengths of acetic acid were used.

In two experiments a series of mixtures of sodium acetate and acetic acid was used, each mixture being 1 to 257 normal with sodium acetate. Such solutions are not generally to be recommended. In one other case the salt content was different (table III, footnote 12).

Young cultures were produced by inoculating broth flasks taken cold from the refrigerator and by placing the flasks in the thermostat at 37.5° C. for eighteen hours or less. Cultures thus obtained showed well staining Gram-positive diplococci and short chains of good form. Except in experiment III such young cultures were always used for the tests. All cultures used were carefully examined and found pure. The cultures were whirled at high speed in a centrifuge and the supernatant fluid was poured off, leaving the deposit of cocci with only a small amount of adhering fluid. The cocci were then well emulsified in a volume of distilled water equal to about one tenth of that occupied by the original culture. 0.3 of a cubic centimeter of this emulsion was placed in each of a series of tubes in a rack, and one cubic centimeter of the proper reaction mixture of the regulator to be employed was added to each tube. Tubes containing acetic acid were stoppered. The rack of tubes was then shaken a few times in a standardized manner, because it was found that shaking markedly aids the agglutination, and was then placed in a water bath at about 37.5° C. until agglutination occurred.

#### EXPERIMENTATION.

The writer found at once that the strains of pneumococcus at his disposal were not readily agglutinated by the acid-salt mixtures (acetic acid, also lactic acid) generally used for the test. Some strains showed no agglutination even after several days at body temperature. Michaelis states that the presence of salts inhibits the acid agglutination of bacteria, but that the effect of salts is slight in concentrations below 1/40 normal. Other investigators apparently have not studied this matter further. The following experiment was performed to see whether in the case of pneumococcus salts can be active in unusually low concentrations.

*Acid Agglutination of Pneumococci.*

## EXPERIMENT I.

*Inhibiting Action of Salts in Low Concentrations.  
Pneumococcus I.*

Regulator.	Salt content.	Mixtures incubated.	Ratio acid : salt (lactate).								
			1/8	1/4	1/2	1	2	4	8	16	32
Lactates.....	N/52	21 hrs.	-	-	-	-	-	-	-	-	-
Lactates diluted 1:6....	N/312	3 hrs.	-	-	-	+	++	+	-	-	-
Equivalent acetic acid ..	None added	3 hrs.	-	-	-	-	++	++	-	-	-

*Pneumococcus 29.*

Regulator.	Salt content.	Mixtures incubated.	Ratio acid : salt (lactate).								
			1/8	1/4	1/2	1	2	4	8	16	32
Lactates.....	N/52	21 hrs.	-	-	-	-	-	-	-	-	-
Lactates diluted 1:6....	N/312	21 hrs.	-	-	-	-	-	-	-	-	-
Equivalent acetic acid ..	None added	3 hrs.	-	-	-	-	-	-	-	+	-
		21 <sup>2</sup> hrs.							+	+	+

This experiment shows plainly that the pneumococcus is sensitive to the action of salts in inhibiting acid agglutination even at a normality of 1/312 of salt. This was seen many times in later experimentation.

## EXPERIMENT II.

*Effect of Washing Pneumococci in Distilled Water.**Pneumococcus 75.*

All Mixtures Were Incubated for Twenty-Eight Hours.

		Ratio acid : salt (lactate).								
		1/8	1/4	1/2	1	2	4	8	16	32
Lactate mixture .....										
Unwashed.....		-	-	-	-	-	-	-	-	-
Washed.....		-	-	-	-	-	+	-	-	-
Lactates diluted 1:2.5.....		-	-	-	-	-	+	-	-	-
Unwashed.....		-	-	-	-	-	+	-	-	-
Washed.....		-	-	-	-	-	+	-	-	-
Lactates diluted 1:6.....		-	-	-	-	-	+	-	-	-
Unwashed.....		-	-	-	-	+	+	+	-	-
Washed.....		-	-	-	+	+	+	+	-	-
Equivalent acetate mixtures.....		-	-	-	+	+	+	+	+	-
Unwashed.....		-	-	-	+	+	+	+	+	-
Washed.....		-	-	-	+	++	++	+	+	-

The technique was now controlled to see whether washing the deposit of pneumococci would be a real refinement.

<sup>2</sup> Autolysis took place in the first three tubes during this time.

This experiment shows that no less acid is required to produce a given result<sup>8</sup> when the cocci are washed. Unwashed, though well drained, bacterial deposits were therefore used for all the tests that follow.

Certain results obtained by Dr. Hanes in this laboratory, when compared with the results of the writer, suggested that cultures incubated a few hours longer than was the practice in this investigation agglutinated well in the presence of the usual amount of salt, but at less acid reactions. A flask of broth was therefore inoculated with pneumococcus I and from it in twenty-four hours material was taken and inoculated into a second flask. Both flasks were then incubated for about eighteen hours. Smears showed that the old culture contained many Gram-negative as well as Gram-positive diplococci, whereas the young culture contained only Gram-positive organisms. The acid agglutination test, with undiluted lactates, gave the following results.

### EXPERIMENT III.

#### *Age of the Culture.*

Culture.	Mixtures incubated.	Ratio acid : salt.					
		2	4	8	16	32	
Young . . .	6 hrs.	-	-	-	-	-	-
Young . . .	20 hrs.	-	-	-	-	-	-
Old . . . .	1 hr.	-	-	-	-	-	-
Old . . . .	2 hrs.	-	-	+	-	-	-
Old . . . .	6 hrs.	-	+	+	-	-	-
Old . . . .	20 hrs.	-	+ =	++	-	-	-

It will be seen that the old culture agglutinates well in six hours, though the young culture shows nothing in twenty hours, but that the optimum in the case of the old culture is different from that characteristic of the strain and of other organisms of the type (experiment I and table I). The optimum seen here for the old culture coincides with that which Beniasch found for the pneumococcus.

With the technique given above, various strains of pneumococci were now examined by the method of acid agglutination. In accordance with a classification already made for these strains by the

<sup>8</sup> The power of acid regulators to maintain the calculated hydrogen ion concentration in the presence of traces of medium decreases with decreasing concentration of acid in the regulating mixture.

*Acid Agglutination of Pneumococci.*

use of immune sera, by protection of mice by the simultaneous administration of serum with living coccii and by agglutination (Dochez and Gillespie (5)), the material is divided into three groups and presented in tables I, II, and III. Many of the strains were at once tested with three regulators (lactates, lactates 1 to 6, and acetic acid). All strains were tested at least once with the undiluted lactates, and when the result was negative a lower salt concentration was tried by the use of the diluted lactates or acetic acid. No strain failed to agglutinate with the acetic acid mixture. Negative results, which were always attributable to too high a salt content of the regulator, have not been recorded in the tables.

TABLE I.  
*Acid Agglutination of Pneumococci of Type I (Neufeld 1).*

Culture. <sup>4</sup>	Regulator. <sup>5</sup>	Hours. <sup>6</sup>	Hydrogen ion concentration. <sup>7</sup>							
			0.2	0.4	0.7	1.4	2.8	5.5	11.0	22.0
1.58 <sup>12</sup>	Lactate 1:6	45	-	-	-	-	-	±	±	-
1.58 <sup>16</sup>	Lactate 1:6	2	-	-	-	-	-	+	-	-
1.58 <sup>16</sup>	Acetic acid	2	-	-	-	-	-	+	+	-
1.64 <sup>7</sup>	Lactate 1:6	3	-	-	-	-	+	++	±	-
1.64 <sup>7</sup>	Acetic acid	3	-	-	-	-	-	++	++	-
1.67 <sup>7</sup>	Acetic acid	1 1/2	-	-	-	-	-	+	+	-
88.0 <sup>18</sup>	Lactate	6 1/2	-	-	-	-	-	±	±	-
75.6 <sup>8</sup>	Lactate 1:6	28	-	-	-	-	±	+	±	-
75.6 <sup>8</sup>	Acetate	28	-	-	-	-	±	+	+	±
94.1 <sup>7</sup>	Lactate	50	-	-	-	-	+	+	-	-
94.1 <sup>7</sup>	Lactate 1:6	4	-	-	-	-	-	++	±	-
43.5 <sup>19</sup>	Lactate 1:6	5 1/2	-	-	-	-	-	+	++	-
43.5 <sup>19</sup>	Acetic acid	1 3/10	-	-	-	-	-	+	++	-
73.1 <sup>10</sup>	Lactate 1:6	20	-	-	-	-	-	±	+	-
73.1 <sup>10</sup>	Acetic acid	20	-	-	-	-	-	±	++	-
73.1 <sup>18</sup>	Acetic acid	1 3/10	-	-	-	-	-	+	++	-
90.1 <sup>10</sup>	Lactate 1:6	1 3/10	-	-	-	-	-	±	±	-
90.1 <sup>10</sup>	Acetic acid	1 3/10	-	-	-	-	-	+	++	-
90.1 <sup>11</sup>	Lactate 1:6	1 1/2	-	±	+	+	+	+	-	-
90.1 <sup>11</sup>	Acetic acid	1 1/2	-	±	+	+	+	+	-	-
44.6 <sup>18</sup>	Lactate 1:6	1 1/5	-	-	-	-	++	+	-	-
44.6 <sup>18</sup>	Acetic acid	1 1/5	-	-	-	-	++	++	-	-

<sup>4</sup> The integral part of the laboratory number specifies the strain, the decimal gives the number of animal passages, and the exponent gives the number of transfers since the last animal passage.

<sup>5</sup> These regulators have been described above. Lactate means the sodium lactate-lactic acid mixtures; lactate 1:6 means such mixtures freshly diluted six times with distilled water; acetate means the sodium acetate-acetic acid mixtures.

<sup>6</sup> Time required for the recorded degree of agglutination.

<sup>7</sup> Multiplied by 10<sup>4</sup>. The unit is a gram per liter.

In table I are given the results for eight strains of pneumococci of the No. 1 (or Neufeld) type. Only two positive results, and these were weak, were obtained with the undiluted lactate mixtures. This type shows narrow zones of agglutination with an optimum at a hydrogen ion concentration of 5.5 to 11 times  $10^{-4}$  grams per liter. This optimum is therefore a little different from that found by Beniasch (2) for pneumococci.

TABLE II.  
*Acid Agglutination of Pneumococci of Type 2.*

Culture.	Regulator.	Hours.	Hydrogen ion concentration. <sup>8</sup>								
			0.2	0.4	0.7	1.4	2.8	5.5	11.0	22.0	44.0
2.10 <sup>6</sup>	Acetic acid	2 3/5				—	—	—	—	+	±
2.10 <sup>17</sup>	Acetic acid	1 1/2				—	—	—	+	+	—
95.0 <sup>9</sup>	Lactate	17	—	—	—	—	—	—	±	±	±
95.0 <sup>7</sup>	Lactate 1:6	50	—	—	—	—	—	±	+	+	+
85.6 <sup>9</sup>	Lactate 1:6	4	—	—	—	—	—	—	+	—	—
85.6 <sup>11</sup>	Lactate 1:6	20	—	—	—	—	—	±	±	+	±
85.6 <sup>11</sup>	Acetic acid	2	—	—	—	—	—	—	+	+	—
85.6 <sup>12</sup>	Acetic acid	3	—	—	—	—	—	—	+	—	—
(Same, read later)		22	—	—	—	—	—	—	++	+	±
15.7 <sup>12</sup>	Acetic acid	1	—	—	—	—	—	—	+	+	—
23.6 <sup>11</sup>	Acetic acid	1	—	—	—	—	—	—	±	±	—
39.5 <sup>8</sup>	Acetic acid	5 1/2	—	—	—	—	—	—	++	+	—
29.7 <sup>12</sup>	Acetic acid	3	—	—	—	—	—	—	—	+	—
89.1 <sup>9</sup>	Lactate	1	—	—	+	+	+	+	+	±	—
89.1 <sup>9</sup>	Lactate 1:6	1	—	—	+	+	+	+	+	±	—
89.1 <sup>9</sup>	Acetic acid	1	—	—	±	+	±	+	+	±	—
89.2 <sup>9</sup>	Lactate	1/5	—	—	±	±	±	+	+	+	±
89.2 <sup>9</sup>	Lactate	1/5	—	±	±	±	±	+	+	+	+

Table II gives the results for eight strains of pneumococci of a different serological type (No. 2). With the exception of No. 89 these strains showed weak or no agglutination with the undiluted lactate mixture. Some even gave negative results with the diluted lactates. With the same exception the strains show a narrow zone of agglutination with an optimum of 11 to 22 times  $10^{-4}$  grams of hydron per liter. This lies within the region found by Beniasch to be characteristic for streptococci.

<sup>8</sup> Multiplied by  $10^4$ .

<sup>9</sup> Pneumococcus 89 has constantly shown atypical forms in cultures. Its behavior with serum 2 is, however, perfectly typical.

*Acid Agglutination of Pneumococci.*

TABLE III.  
*Acid Agglutination of Atypical Strains of Pneumococci.*

Culture.	Regulator.	Hödrs.	Hydrogen ion concentration. <sup>10</sup>									
			0.04	0.1	0.2	0.4	0.7	1.4	2.8	5.5	11.0	22.0
71.1 <sup>10</sup>	Lactate	17									+	+
71.1 <sup>10</sup>	Lactate 1:6	1									+	+
71.1 <sup>10</sup>	Acetic acid	1									+	+
38.6 <sup>11</sup>	Lactate	1 1/5								+	+	+
38.6 <sup>11</sup>	Lactate 1:6	1 1/5								+	+	+
36.16 <sup>12</sup>	Lactate	1								+	+	+
36.16 <sup>12</sup>	Lactate 1:6	1								+	+	+
36.16 <sup>12</sup>	Acetic acid	1								+	+	+
55.6 <sup>11</sup> <sup>11</sup>	Lactate	1 1/5								+	+	+
55.6 <sup>11</sup>	Lactate 1:6	1 1/5								+	+	+
PaS.1 <sup>2</sup>	Lactate	1 1/2								+	+	+
PaS.1 <sup>2</sup>	Acetic acid	1 1/2								+	+	+
62.0 <sup>8</sup>	Lactate	2								+	+	+
62.0 <sup>8</sup>	Acetic acid	2								+	+	+
37-8.6 <sup>13</sup>	Lactate	1 1/5								+	+	+
37-8.6 <sup>13</sup>	Acetic acid	1 1/5								+	+	+
34.6 <sup>15</sup>	Lactate	22								+	+	+
34.6 <sup>17</sup>	Lactate	45		-	±	+				-	-	-
76.4 <sup>11</sup>	Lactate	2								+	+	+
76.4 <sup>11</sup>	Acetic acid	2								+	+	+
76.4 <sup>11</sup>	Lactate	1 3/10		+	++	+±				+	+	+
82.5 <sup>9</sup>	Lactate	22								+	+	+
82.5 <sup>11</sup>	Lactate	1 1/2								+	+	+
52.2 <sup>9</sup>	Lactate	6 1/2								+	+	+
52.2 <sup>13</sup>	Lactate	1 3/10	+	+	+±	+				+	+	+

*Streptococcus mucosus.*

49.3 <sup>9</sup>	Lactate	2		+±	±	-	-	-	-	-	-	-
19.9 <sup>5</sup>	Acetate <sup>12</sup>	1 8/10		-	++	+++	+++	++	-	-	-	-

Table III contains the results for ten strains belonging to neither type 1 nor 2, and not showing any considerable degree of relationship one to another by serum tests; for one strain (No. 55) which showed relationship with type 2 by the protection test, but not by the agglutination test; and for two strains of *Streptococcus mucosus*, one of which (No. 49) has all the properties of a streptococcus except that it has a large capsule and produces a mucous exudate in mice, the other of which (No. 19) has all the typical properties of a pneumococcus except for its extra large capsule, mucoid growth on

<sup>10</sup> Multiplied by 10<sup>4</sup>.

<sup>11</sup> *Pneumococcus* 55 belongs with type 2 by protection test, but it failed to agglutinate with serum 2.

<sup>12</sup> The concentration of sodium acetate was in this case N/26.

agar, and production of a mucous exudate in mice. The agglutination of most of these organisms is rapid and is never inhibited by salts in the same manner as is that of pneumococci of types 1 and 2. The zones of agglutination are for the most part broad; none are coincident with those characteristic of types 1 and 2.

#### SUMMARY.

Eight strains of pneumococci of serological type 1, eight strains of type 2, and eleven strains belonging to neither type have been tested by the method of acid agglutination.

Strains belonging to the two typical groups have, as a rule, narrow zones of agglutination. The optimum hydrogen ion concentrations are different in the two cases. Other pneumococci have broad zones or, in a few cases, narrow zones not coincident with those occupied by the typical organisms.

The agglutination of most of the pneumococci of types 1 and 2 is extremely susceptible to the inhibiting action of salts. This is not true of the other pneumococci.

Old broth cultures may show an optimum hydrogen ion concentration different from that shown by young broth cultures.

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## AN IMMUNOLOGICAL STUDY OF PNEUMOCOCCUS MUCOSUS.\*

By FREDERIC M. HANES, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Although the older literature, reviewed by von Lingelsheim (1), contains a number of descriptions of encapsulated streptococci, the importance of organisms of this type as frequent producers of disease has only been fully recognized since the publications of Schottmüller (2), who found a well characterized organism, which he called *Streptococcus mucosus*, in various pyogenic processes and in lobar pneumonia. Many observers have since encountered this organism, occurring as it does in the most diverse pyogenic lesions, and its morphologic and biologic characteristics are well known.

Among seventy-four cultures isolated from patients with lobar pneumonia in the Hospital of The Rockefeller Institute in the course of the past two years, nine, or 12 per cent., have been classed provisionally as belonging to the group of encapsulated streptococci of Schottmüller. These organisms, through their power of producing a tenacious mucoid exudate in the peritoneal cavity of white mice, were readily separated from the larger group of encapsulated diplococci. At the same time, however, their cultural characteristics made it seem probable that they represented a variety of the pneumococcus rather than that they belonged to the streptococcus group.

With the hope of determining more exactly the relations of *Streptococcus mucosus* of Schottmüller to *Diplococcus pneumoniae*, on the one hand, and to the streptococci, on the other, six of our nine organisms were selected for a comparative study of this problem. These six organisms were chosen at random from the nine in our possession. For convenience I shall refer to this group of organisms as *Pneumococcus* rather than *Streptococcus mucosus*. They were reobtained for this study from the dried spleens<sup>1</sup> of white mice which had died following a peritoneal injection of the sputum from

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<sup>1</sup> It not infrequently happens that mice infected intraperitoneally with pneumococci show, in addition to a pneumococcus septicemia, an infection with the

patients suffering from lobar pneumonia, the spleens having been preserved *in vacuo* for several months (Neufeld and Händel (3)). Two other pneumococci and two organisms of the streptococcus group were selected for study in addition to these six. The distinguishing characteristics of all the organisms are given for comparison in table I.

The six representatives of the *Pneumococcus mucosus* group (Nos. 19, 26, 42, 54, 68, and 96) possess identical properties, and these will be described in detail. Of the two other pneumococci, one is an organism secured from Dr. Neufeld and is the type representative of Dochez's (4) group I; the other belongs to Dochez's group II. One of the streptococci (No. 174) is a typical hemolyzing *Streptococcus pyogenes*, while the other (No. 49) is exactly described by the name *Streptococcus mucosus*. This organism was selected for comparative study because it possesses a distinct capsule and has the power of producing a mucoid exudate in the peritoneal cavity of a white mouse, having at the same time the cultural and morphological characters of a streptococcus. Its characteristics, as detailed in table I, would seem to place it nearer the streptococci than the pneumococci. It was recovered from the sputum of a non-fatal case of lobar pneumonia.

As stated above, the six members of the group of *Pneumococcus mucosus* chosen for this study exhibit identical properties; they likewise conform to Schottmüller's description of *Streptococcus mucosus*. There can be no doubt, then, that they belong to the same group as the organisms he described, and a brief description of this group will be given.

*Pneumococcus mucosus* occurs in diplococcal form, often arranged in short chains of from four to about sixteen members. These are surrounded by a broad mucoid capsule which, in purulent exudates, bacillus of mouse typhoid (*B. typhi murium*). Both organisms are often present in the blood, but this may show only pneumococci, while smears of the exudate from the peritoneal cavity show that both are present. Under such conditions the dried spleens in which the pneumococci are preserved are contaminated with *B. typhi murium*, and the recovery of the pneumococcus in pure culture is very difficult. Several of our spleens were thus contaminated, and other methods of purification having failed, we resorted with complete success to the production in rabbits of an anti-mouse typhoid serum. These sera will prevent the growth of the mouse typhoid in the animal body, whereas pneumococci develop as usual.

*Immunological Study of Pneumococcus mucosus.*

TABLE I.

Organism.	Morphology.	Solubility in medium tauroholate.	Growth on blood agar plates.	Plain agar.	Litmus-milk.	Bouillon.	Hisa's serum inulin medium.	Exudate in peritoneal cavity of mice.
Six strains of <i>Pneumococcus mucosus</i> (Nos. 19, 26, 42, 54, 68, 96)	Diplococci single and in short chains; cocci round or slightly flattened, and possessing broad, mucoid capsules	Large round Soluble	Very delicate, translucent, translucent colonies, colorless and translucent; greenish brown discoloration of medium	Acid and coagulated in 24 hrs.	Moderately turbid; very slight sediment	Acid and coagulated in 24 hrs.	Mucoid, tenacious, easily drawn out into thread	
Pneumococcus I and II	Lance-shaped diplococci with delicate capsules	Soluble	Delicate, discrete, drop-like colonies; greenish brown discoloration of medium	Acid and coagulated in 24 hrs.	Very delicate, translucent, translucent growth; media not clouded	Moderately turbid; very slight sediment	Acid and coagulated in 24 hrs.	Not mucoid or tenacious.
Organism 49. <i>Streptococcus mucosus</i>	Small round cocci arranged in short or long chains	Not soluble	Grayish translucent colonies	A bundle of acid and coagulated in 24 hrs.	Turbid; moderately heavy sediment	No acid production or coagulation	Mucoid and tenacious.	
<i>Streptococcus longus</i>	Small round cocci arranged in chains	Not soluble	Grayish translucent colonies; wide area of hemolysis	Abundant small acid and coagulated in 24 hrs.	Turbid; No acid sediment	No acid production or coagulation	Not mucoid or tenacious.	

merges almost imperceptibly into the characteristic tenacious material produced so abundantly by the growth of this organism in the animal body. The capsule persists in cultures upon artificial media and is always easily demonstrated. The organism is round or slightly flattened in shape and varies in size, even in the same culture. It gives the impression, especially when stained by Gram's method, of being larger and coarser than other pneumococci.

*Pneumococcus mucosus*, in common with other pneumococci, grows best upon a medium containing blood serum or whole blood. Growth is very scanty or absent upon plain agar, while relatively few of the transplanted organisms grow even upon a favorable medium such as blood agar. It is necessary, in order to secure luxuriant growth, to inoculate heavily any medium used. The cultures dry out rapidly, even under the favorable conditions of ice box temperature, and after ten days they are usually sterile. The individual colonies are round, moist, colorless, and translucent. When several colonies have become confluent, as they do very readily, they tend to run down the surface of the agar slant, presenting the appearance of a dependent drop of clear, tenacious, mucoid material. Smear cultures on blood agar present, as a rule, a smooth, homogeneous, translucent growth, which is colorless itself, but causes a greenish brown discoloration of the medium. With the platinum loop, the growth can be drawn out into short tenacious threads. Bouillon, if carefully prepared and heavily inoculated, produces a moderately luxuriant growth, with little or no tendency to sedimentation. Growth on gelatin is slight and there is no liquefaction. Litmus-milk turns red and coagulates solidly in twenty-four hours. Hiss's serum inulin medium is reddened and coagulated.

The organism dissolves readily in the presence of a solution of sodium taurocholate. If several drops of a 2 per cent. solution of sodium taurocholate are added to two cubic centimeters of a twenty-four hour growth in bouillon, the mixture rapidly becomes clear, provided there is no trace of blood serum present.

*Pneumococcus mucosus* is highly pathogenic for white mice and rabbits. One millionth of a cubic centimeter of an eighteen hour bouillon culture will kill a mouse in from fifteen to twenty-four hours when injected intraperitoneally. Its pathogenicity does not seem to be lessened by prolonged cultivation upon artificial media.

## SPECIFIC AGGLUTINATION OF PNEUMOCOCCUS MUCOSUS.

It has been found by Dochez and Gillespie (4) that, by means of immunological reactions (agglutination and protection), the various strains of pneumococci recovered from the sputum and blood of patients with lobar pneumonia can be classified into at least three groups, with a fourth, less well defined group comprising organisms showing no interrelations. Their results demonstrate beyond question the heterogeneous character of the group of organisms possessing the physical and cultural properties of the pneumococcus. The macroscopic agglutination method was employed by Dochez, equal quantities of immune serum and twenty-four hour bouillon culture being used in the reaction. The results obtained were clean cut and definite, and were fully confirmed in each instance by the results of protection experiments, so that notwithstanding the low serum dilution employed, the method yielded specific information.

This method was therefore employed in attempting to demonstrate specific agglutinins for *Pneumococcus mucosus* in the serum of rabbits highly immunized to this organism. My results were uniformly negative. Two possibilities presented themselves. Either *Pneumococcus mucosus* was incapable of stimulating specific agglutinins, or else the method employed for their detection was inadequate. The first possibility seemed unlikely, since rabbits could be brought to a high state of active immunity to *Pneumococcus mucosus*,—higher indeed than rabbits immunized to other types of pneumococci and yielding good, specifically agglutinating sera.

A distinct analogy seemed to exist between the problem here presented and that of the specific agglutination of encapsulated bacilli; for the heavy mucoid capsule of *Pneumococcus mucosus* is its most strikingly distinguishing feature when compared with other pneumococci. The work of Porges (5) and von Eisler and Porges (6) has demonstrated that, contrary to the experience of previous workers, the capsule bacilli can be agglutinated readily by homologous immune sera, provided the bacilli are subjected previously to a preparatory treatment directed toward the destruction of their capsules.

The procedure recommended for this purpose by Porges has been employed with excellent results in the present attempt to demonstrate specific agglutinins in animals immunized to *Pneumococcus mucosus*. The method of Porges, as adapted to the present study, is as follows: Half a liter of a twenty-four hour bouillon culture of *Pneumococcus mucosus* was centrifugalized at high speed for half an hour, the supernatant fluid decanted off, and the organisms taken up in ten cubic centimeters of salt solution. Three cubic centimeters of this emulsion were added to each of three test-tubes and one cubic centimeter (one fourth volume) of N/4 hydrochloric acid was added to each. The tubes were placed in a water bath at 80° C., and one was removed in twenty minutes, the second in thirty, and the third in fifty minutes. They were placed immediately in ice water, and when thoroughly cooled one cubic centimeter of N/4 sodium hydrate was added to each tube. The emulsions thus prepared are neutral, they show no tendency toward spontaneous agglutination, and sedimentation takes place very slowly.

The six *Pneumococcus mucosus* organisms were treated after this fashion and the agglutinability of each was tested with the sera of six rabbits, each of which had been immunized to one of these organisms. The results are shown in table II.

TABLE II.  
*Agglutination (Porges Method).*

Organism No.	Immune sera.										Normal rabbit serum.
	19	26	42	54	68	96	I	II	49	174	
19	+	+	+	+	+	+	-	-	-	-	-
26	+	+	+	+	+	+	-	-	-	-	-
42	+	+	+	+	+	+	-	-	-	-	-
54	+	+	+	+	+	+	-	-	-	-	-
68	+	+	+	+	+	+	-	-	-	-	-
96	+	+	+	+	+	+	-	-	-	-	-
I	-	-	-	-	-	-	+	-	-	-	-
II	-	-	-	-	-	-	-	+	-	-	-

In every instance the treated organisms agglutinated, whereas twenty-four hour bouillon cultures of the same organisms constantly failed to agglutinate. Equal quantities of immune serum and emulsion were used, and the tubes were kept at 37° C. during the experiment. Agglutination appeared promptly as a rule, and readings

made at the end of one hour were usually positive; final readings were made after six hours. The end result is quite definite, the supernatant fluid being clear, with the organisms forming a loosely coherent mass at the bottom of the tube. Of the three tubes of emulsion which were heated for varying lengths of time, the one treated for fifty minutes agglutinated slightly better than the others. As controls, emulsions of two other pneumococci (Nos. I and II) were made as described above, and these were likewise treated with the six *Pneumococcus mucosus* sera and with their own specific immune sera. They failed to agglutinate with the former, although with its own specific serum each agglutinated promptly. The emulsions of *Pneumococcus mucosus* were in no case agglutinated by sera other than their own, and remained well suspended in normal sera.

These reactions leave no room for doubt that the method of Porges is efficacious in rendering *Pneumococcus mucosus* specifically agglutinable. This result is probably though not certainly attributable to the destruction of the heavy, mucoid capsules of the organisms. It is conceivable that other factors play a part but, at any rate, it can easily be demonstrated that the capsules have almost if not quite disappeared during the treatment.

#### COMPLEMENT FIXATION EXPERIMENTS.

It is desirable to learn not only the relations of *Pneumococcus mucosus* to other pneumococci, but to the streptococcus group as well. Agglutination experiments demonstrated clearly the presence of specific agglutinins in the sera of rabbits immunized with *Pneumococcus mucosus*, but since the method employed is inapplicable to the streptococci, owing to their marked tendency toward spontaneous clumping and sedimentation, it throws no light upon a possible relationship of *Pneumococcus mucosus* to the streptococcus group.

The complement fixation method of Bordet-Gengou seemed much better adapted to this purpose and was therefore used with the results detailed below. The slightly modified Wassermann technique, as described by Walker and Swift (7), was employed.

*Antigens.*—Antigens with strong fixing power and weak anti-complementary action are readily obtained with *Pneumococcus*

*mucosus* (8). Twenty-four hour bouillon cultures are thoroughly centrifuged, the organisms washed once in normal salt solution, this removed as completely as possible, and the residue dried *in vacuo* over sulphuric acid. The dried substance is now weighed, one cubic centimeter of salt solution containing 0.5 per cent. phenol is added for each milligram of the dried material, and the whole is shaken for twenty-four hours in a bottle containing glass beads. The resulting emulsion is centrifugalized at high speed and the clear or slightly opalescent supernatant fluid is pipetted into rubber-corked brown bottles and kept in the ice box. Under these conditions the antigens have shown no deterioration after more than three months.

Antigens were prepared from each of the six strains of *Pneumococcus mucosus* and from pneumococcus I and II. In addition, antigens were made in a similar fashion from the *Streptococcus mucosus* and *Streptococcus pyogenes* previously described.

*Immune Sera*.—Rabbits were immunized to all the various organisms mentioned above. *Pneumococcus mucosus* is highly pathogenic for rabbits and, to avoid losses, the greatest care is necessary in rendering them immune to it. It frequently happens that rabbits succumb to a dose only slightly larger than the previous one, even after weeks of treatment with carefully graded doses of the organism. With care, however, quite high degrees of active immunity can be obtained, so that animals will show no serious reaction after an intravenous injection of the bacteria from 100 cubic centimeters of twenty-four hour bouillon culture. When we recall that 0.00001 of a cubic centimeter will kill, this degree of immunity is seen to be considerable. Organisms should always be centrifugalized free from the bouillon medium, for it is harmful when injected repeatedly. It has been found best to give an initial injection of killed organisms from 30 to 50 cubic centimeters of bouillon, and then to use live organisms for the next injection, beginning with very small doses and increasing as rapidly as the animal's condition permits. It is desirable that the animals should react severely to the injections; those animals in my series which have shown high fever and considerable loss of weight have furnished the most powerful sera.

Guinea pig complement, sheep cells, and anti-sheep cell rabbit serum were employed as a hemolytic system.

The results of the complement fixation experiments are illustrated by the tables III to X, which have been selected as typical. The various immune sera were first tested against constant quantities (0.1 of a cubic centimeter) of the different antigens, the sera being progressively diluted, and then against diminishing quantities of antigens, 0.1 of a cubic centimeter of serum being used.

The amount of antigen used was never greater than one fifth of the anticomplementary dose. The uniformity in the strength of antigens prepared from the six strains of *Pneumococcus mucosus* has been striking. As an antigen, any one of them could be chosen as representative of the group.

Specifically fixing sera have been obtained, as a rule, without great difficulty. It has been easier to obtain sera with high fixing powers by immunizing with *Pneumococcus mucosus* than with the other types of organisms used. This may be due in part to the severe reactions which the *Pneumococcus mucosus* animals showed to the earlier injections.

TABLE III.

*Serum 26 in Diminishing Quantities with Constant Amount (0.1 Cubic Centimeter) of Antigen.*

Antigen No.	0.005 c.c.	0.0025 c.c.	0.0012 c.c.	0.0006 c.c.	0.0003 c.c.
26 (0.1 c.c.)	+++	+++	+++	++	+
42 (0.1 c.c.)	+++	+++	++	+	-
96 (0.1 c.c.)	+++	+++	++	+	-
I (0.1 c.c.)	+	+	-	-	-
II (0.1 c.c.)	+	#	-	-	-
49 (0.1 c.c.)	-	-	-	-	-
174 (0.1 c.c.)	-	-	-	-	-

TABLE IV.

*Serum 68 in Diminishing Quantities with Constant Amount (0.1 Cubic Centimeter) of Antigen.*

Antigen No.	0.005 c.c.	0.0025 c.c.	0.0012 c.c.	0.0006 c.c.	0.0003 c.c.
68 (0.1 c.c.)	+++	+++	++	+	+
19 (0.1 c.c.)	++	++	#	-	-
26 (0.1 c.c.)	+++	+++	++	+	-
42 (0.1 c.c.)	+++	+++	++	+	#
54 (0.1 c.c.)	+++	+++	++	+	-
96 (0.1 c.c.)	+++	+++	++	++	+
I (0.1 c.c.)	++	+	-	-	-
II (0.1 c.c.)	+	#	-	-	-
49 (0.1 c.c.)	-	-	-	-	-
174 (0.1 c.c.)	-	-	-	-	-

TABLE V.

*Serum 96 in Diminishing Quantities with Constant Amount (0.1 Cubic Centimeter) of Antigen.*

Antigen No.	0.005 c.c.	0.0025 c.c.	0.0012 c.c.	0.0006 c.c.	0.0003 c.c.
96 (0.1 c.c.)	+++	+++	+++	+++	+++
19 (0.1 c.c.)	+++	+++	++	+ $\pm$	-
26 (0.1 c.c.)	+++	+++	+++	+++	+++
42 (0.1 c.c.)	+++	+++	+++	+++	++
54 (0.1 c.c.)	+++	+++	+++	+++	++
68 (0.1 c.c.)	+++	+++	++	+	+
I (0.1 c.c.)	++	++	-	-	-
II (0.1 c.c.)	+	$\pm$	-	-	-
49 (0.1 c.c.)	-	-	-	-	-
174 (0.1 c.c.)	-	-	-	-	-

TABLE VI.

*Serum I in Diminishing Quantities with Constant Amount (0.1 Cubic Centimeter) of Antigen.*

Antigen No.	0.05 c.c.	0.01 c.c.	0.005 c.c.	0.0025 c.c.
I	+++	+++	++	+
II	+	+	-	-
19	++	++	+	-
26	+++	++	+	-
42	+++	+++	++	+
68	++	++	+	-
96	+++	+++	++	-
49	-	-	-	-
174	-	-	-	-

TABLE VII.

*Serum II in Diminishing Quantities with Constant Amount (0.1 Cubic Centimeter) of Antigen.*

Antigen No.	0.05 c.c.	0.01 c.c.	0.005 c.c.	0.0025 c.c.
II (0.1 c.c.)	+++	++	+	-
I (0.1 c.c.)	++	+	-	-
19 (0.1 c.c.)	++	+	-	-
26 (0.1 c.c.)	++	+	-	-
42 (0.1 c.c.)	++	++	-	-
68 (0.1 c.c.)	++	+	-	-
96 (0.1 c.c.)	++	+	-	-
49 (0.1 c.c.)	-	-	-	-
174 (0.1 c.c.)	-	-	-	-

TABLE VIII.

*Serum 26, 0.1 Cubic Centimeter, with Decreasing Amounts of Antigen.*

Antigen No.	0.05 c.c.	0.04 c.c.	0.03 c.c.	0.02 c.c.	0.01 c.c.
26	+++	+++	+++	++	-
19	+++	++	+	±	-
42	+++	+++	++	+	+
54	+++	+++	++	+	-
68	+++	+++	+++	+	-
96	+++	++	++	+	-
I	++	+	-	-	-
II	+	-	-	-	-

TABLE IX.

*Serum 42, 0.1 Cubic Centimeter, with Decreasing Amounts of Antigen.*

Antigen No.	0.05 c.c.	0.04 c.c.	0.03 c.c.	0.02 c.c.	0.01 c.c.
42	+++	+++	++	+	+
19	++	++	+	-	-
26	+++	+++	++	+	-
54	+++	+++	+	±	-
68	+++	+++	++	+	+
96	+++	+++	++	+	-
I	++	++	-	-	-
II	+	-	-	-	-

TABLE X.

*Serum 96, 0.1 Cubic Centimeter, with Decreasing Amounts of Antigen.*

Antigen No.	0.05 c.c.	0.04 c.c.	0.03 c.c.	0.02 c.c.	0.01 c.c.
96	+++	+++	+++	++	+
19	+++	+++	++	+	-
26	+++	+++	+++	++	+
42	+++	+++	+++	++	+
68	+++	+++	+++	++	+
I	++	++	++	+	-
II	+	+	-	-	-

As shown in the tables, there has been a certain amount of cross-fixation, or group reaction, among the pneumococci. This group reaction is most clearly demonstrated by using immune sera I and II with *Pneumococcus mucosus* antigens (tables VI and VII). Antigen I has constantly reacted more strongly with *Pneumococcus mucosus* sera than has antigen II. There can be no doubt that, as shown by Dochez, these two organisms are essentially different; they likewise differ in essential points from *Pneumococcus mucosus*.

No cross-fixation was observed in testing the complement-fixing

powers of sera 49 (*Streptococcus mucosus*) and 174 (*Streptococcus pyogenes*). They each fixed specifically with homologous antigens, but showed no fixation with heterologous antigens.

From the foregoing considerations, the conclusion is justifiable that Schottmüller's *Streptococcus mucosus* is in reality a variety of pneumococcus. Its cultural characteristics, solubility in solutions of bile salts, and finally its behavior in comparative complement fixation experiments all support this conclusion.

#### PROTECTION EXPERIMENTS.

The homogeneous character of the *Pneumococcus mucosus* group would, of course, be a most fortunate condition if an antiserum capable of conferring passive immunity could be produced. To determine the possibility of obtaining such a serum has been one of the aims of this study. A high grade of active immunity to *Pneumococcus mucosus* has been produced in rabbits, and the antisera obtained have been used in attempting to protect white mice against this organism. Immune serum and the bouillon culture of the organism were injected simultaneously into the peritoneal cavity. The most favorable result obtained is shown in the following protocol:

Organism 42.	Antiserum 42.	
0.00001 c.c.	0.5 c.c.	Dead in 60 hours.
0.0001 c.c.	0.5 c.c.	Dead in 35 hours.
0.001 c.c.	0.5 c.c.	Dead in 18 hours.
Control.		
0.00001 c.c.		Dead in 18 hours.
0.0001 c.c.		Dead in 15 hours.
0.001 c.c.		Dead in 14 hours.

A slight prolongation of life would seem to have resulted from the use of 0.5 of a cubic centimeter of antiserum, but this result has not been constant with all the sera employed. As a rule the experimental animals and the controls have died within approximately the same lengths of time.

We are confronted, then, with the curious fact that the sera of animals possessing a high degree of active immunity fail to confer passive immunity to homologous organisms upon other animals.

This result is the more surprising because of the relative ease with which protecting antisera can be obtained with other strains of pneumococci. For example, rabbits which have received ten cubic centimeters of a twenty-four hour bouillon culture of pneumococcus I as the maximum immunizing dose, furnish sera which will protect against 0.1 of a cubic centimeter of this organism, whereas the sera of rabbits which have received 100 cubic centimeters of *Pneumococcus mucosus* as a maximum dose fail to protect mice against 0.000001 of a cubic centimeter of the homologous organism. These facts indicate the existence of fundamental differences between *Pneumococcus mucosus* and other varieties of pneumococci, and it is of great interest to inquire into the causes which may possibly underlie these differences.

The failure of *Pneumococcus mucosus* antisera to protect is due in all probability to some peculiarity in the constitution of this organism rendering it extremely resistant to the action of specific antisera, and one naturally thinks first of the heavy mucoid capsule. It is instructive to recall in this connection certain facts which have come to light in the study of the encapsulated bacteria. With these organisms, as with *Pneumococcus mucosus*, no protection has been obtained, although active immunity is produced without difficulty (9). Forges (5) cites an interesting observation upon *Bacillus pneumoniae* of Friedländer. A strain of this organism which had been cultivated for a long period upon artificial media showed no capsule formation and could be agglutinated without previous treatment. It thus seemed a very favorable organism with which to test for specific protection, but when injected into animals together with specific antiserum, no protection was afforded and it was found that the organisms had developed capsules in the animal body. The somewhat similar experiences of Gruber (10) and Löhlein (11) with anthrax and plague bacilli likewise seem to indicate that the serum resistance of certain organisms may be due to capsule formation. As to the correctness of this, judgment must be suspended until more facts are at hand. The problem seems worthy of more careful study than it has as yet received.

Lobar pneumonia caused by *Pneumococcus mucosus* has been in the experience of this hospital a very fatal disease, six of our nine

cases (66 per cent.) having died. The organism was cultivated from the blood in four of the nine cases and none of these recovered.

#### CONCLUSIONS.

1. The organisms described by Schottmüller under the name *Streptococcus mucosus* represent a well defined group with characteristics which indicate a closer relationship to the pneumococci than to the streptococci.
2. The members of this group are specifically agglutinable when treated according to the method of Porges. They do not agglutinate when subjected to the usual agglutination methods.
3. Complement fixation experiments with these organisms, compared with similar experiments with two varieties of pneumococci and two streptococci, indicate that they are closely related to the pneumococci.
4. No protection of mice against *Pneumococcus mucosus* by means of specific immune sera could be demonstrated.
5. The name *Pneumococcus mucosus* should be adopted for this group instead of *Streptococcus mucosus*.

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## ON THE CAUSATION BY FILTERABLE AGENTS OF THREE DISTINCT CHICKEN TUMORS.\*

By PEYTON ROUS, M.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

### PLATES 7 TO 12.

The study of chicken tumors has especial interest because the cause of two such growths has been found in filterable entities. The object of the present paper is to report experiments which show that yet a third neoplasm of the fowl is so caused, and to discuss at some length the methods and findings with all three. One of the growths,—Chicken Tumor I in our series of spontaneous chicken tumors,—is a pure spindle-celled sarcoma;<sup>1</sup> the second (Chicken Tumor VII) is an osteochondrosarcoma;<sup>2</sup> and the third (Chicken Tumor XVIII), of which the cause will here for the first time be reported, is a spindle-celled sarcoma of peculiar intracanalicular pattern.<sup>3</sup> The three are very unlike, not only histologically but in their general behavior. Yet, as will be seen, the entities causing them have much in common and may profitably be considered together.

### THE IMPORTANCE OF PRELIMINARY TRANSPLANTATION.

In our experience the transplantation of chicken tumors is of great importance for experiments looking to their cause. This is in part on account of the material afforded by successful transplantation, but it has its essential basis in the enhanced malignancy resulting therefrom. The findings have shown strikingly that the more malignant the growth the easier is the demonstration of its etiological agent. The fact that tumors caused by an agent dam-

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<sup>1</sup> Rous, P., *Jour. Am. Med. Assn.*, 1911, lvi, 198; *Jour. Exper. Med.*, 1911, xii, 397. For the literature see Rous, P., and Murphy, J. B., *Berl. klin. Wochenschr.*, 1913, I, 637.

<sup>2</sup> Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lix, 1793. Tytler, W. H., *Jour. Exper. Med.*, 1913, xvii, 466.

<sup>3</sup> Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1913, xviii, 651.

aged in some way, as for example by heat or by long sojourn in the dry state, are of relatively benign course<sup>4</sup> suggests that the ready separation of the agent from growths of high malignancy is dependent on its enhanced activity.<sup>5</sup>

#### THE TUMOR-PRODUCING ENTITIES ARE FILTERABLE.

Three methods, namely filtration, desiccation, and glycerination, have been chiefly used to demonstrate a cause for the chicken tumors, as distinct from the tumor cells. Specimen protocols will be given to illustrate the results with each method and these will be followed by a summary of the findings obtained by its employment. The growths produced have in every instance been examined with the microscope.

Filtration has proved by far the most uniformly successful method of study. Under suitable conditions the agents of all the growths will pass through Berkefeld filters that retain small bacteria (*Bacillus prodigiosus*, *Bacillus fluorescens liquefaciens*). Our technique has been described several times. It is again briefly given in the first of the two following protocols that show the filterability of the agent causing Chicken Tumor XVIII.

*Chicken Tumor XVIII, a Sarcoma of Intracanalicular Pattern. Filtration Experiment 1.*—The tumor material came from fowl 257 of the second transplantation generation, series A. Fresh neoplastic tissue to the amount of 7.5 gm. was ground fine with sand, taken up in 250 c.c. of Ringer's solution at 40° C. and a 24 hour culture on slant agar of *Bacillus fluorescens liquefaciens* was added. Shaking was done in a machine for forty-five minutes, then brief centrifugation, and portions of the supernatant fluid were filtered by suction through one or another of three Berkefeld filters; (a) a medium sized V (No. 3), and (b) and (c) two small N cylinders (No. 5). Filtration was continued for about thirty minutes. The fluids from filters a and b were united as filtrate AB. That from filter c will be called filtrate C. To each was added a little sterile diatomaceous earth (*Kieselguhr*) and then 14 c.c. of AB and 7 c.c. of C were injected intramuscularly in the right and left pectoral regions respectively of five normal brown Leghorn fowls. All remained free of tumor until 170 days after the inoculation, when in one a small mass, 2.5 by 1.5 cm. in diameter was noted at the site of injection of filtrate AB. Part of this was removed and found to have

<sup>4</sup> Rous, P., and Murphy, J. B., *Jour. Exper. Med.*, 1913, xvii, 219.

<sup>5</sup> It may not be amiss to point out that in epidemic poliomyelitis, a disease caused by a filterable virus or parasite, the initial infection of monkeys with human nervous tissues is accomplished with difficulty, while after the virus has

the characteristic structure of Chicken Tumor XVIII. The fowl was shortly afterwards lost through accident. The other chickens were kept some months longer but never developed tumors.

Plates of the filtrates (1 c.c. to the tube of agar) remained sterile, whereas plates of the unfiltered fluid (a few drops to the tube of agar) showed innumerable colonies of *Bacillus fluorascens liquefaciens*. At the time of the experiment bits of the tumor which furnished material for it were transplanted into three chickens. Two of these developed tumors within a month. The third remained healthy.

*Filtration Experiment 2.*—The material was furnished by fowl 531 of the third transplantation generation, series C. The tumor was of especially rapid growth. Three Berkefeld filters were employed: (a) a medium sized V cylinder (No. 3), and (b) and (c) two small N cylinders (No. 5). *Bacillus fluorescens liquefaciens* was used as before; and the filters proved impermeable to it. To each of the filtrates, A, B, and C, a little finely ground, sterile, diatomaceous earth was added previous to their separate injection in amounts of 5 c.c. each, into the muscle of the lower leg and breast of eight fowls. Two of the fowls developed progressively growing tumors which appeared, in the one case at sites A and C two months after the time of injection, in the other at sites A and C about five months after it. Both died of the tumor some seven and one half months after inoculation. In addition to the large primary growths there were metastases in the lungs, and in one case secondary growths about several joints (figure 1), a finding not infrequent when Chicken Tumor XVIII is propagated by transplantation. The hip joint was diffusely involved in sarcomatous tissue and fusiform swellings enclosed most of the sternal and vertebral ribs at their junction, forming what might be called a sarcomatous rosary. Whether in the present case these represent primary localizations of the tumor-producing virus or true metastases cannot be said. Microscopically all the growths had the characteristic structure of Chicken Tumor XVIII (figure 2).

In a third fowl a tumor nodule appeared within two months at the site of filtrate B, slowly enlarged to a diameter of 4 cm., and slowly retrogressed again. The only transplantations attempted from the filtrate tumors were performed with this growth. They gave negative results. The other fowls injected with filtrate remained healthy.

The association of a foreign body with the filtrate to bring about a tissue derangement renders much more likely the production of tumors. The influence of the factor has been carefully studied in the case of Chicken Tumor I.<sup>6</sup> In all the experiments we have made use of powdered diatomaceous earth (*Kieselguhr*), which elicits, as Podwyssozki has shown, an intense reactive connective tissue proliferation with the formation of giant cells. Histologically this

been transmitted through several monkeys, it increases markedly in virulence for those animals and can be transferred with far greater certainty.

<sup>6</sup> Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lviii, 1751.

reactive proliferation bears no resemblance to the tumors with which we have worked (figure 10).

*Chicken Tumor XVIII. Filtration Experiment 3.*—Filtrates were prepared in the usual way, through filters which the control with *Bacillus fluorescens liquefaciens* showed to be bacteria-tight. Three Berkefeld cylinders were used: (a) a large unmarked cylinder (No. 2), (b) a medium V (No. 3), and (c) a small N (No. 5). The filtrates were separately examined for the test bacterium, and injected. To half of each was added enough sterile, powdered *Kieselguhr* to give a slight cloudiness. For this very little was required. Six fowls were injected, receiving on one side in the thigh and pectoral muscle respectively two filtrates with *Kieselguhr*, at corresponding points on the other side the same filtrates in equal or slightly greater amount, without *Kieselguhr*. Of the five fowls that survived three months one developed after the sixtieth day small tumor nodules, where the filtrates A and C had been injected in amounts of 10 c.c. and 3 c.c. respectively with *Kieselguhr*. The corresponding sites, where slightly larger quantities of the filtrates without admixture had been put, remained free of growths. The tumors measured 4 cm. and 3 cm. in diameter respectively when the fowl was killed three months after injection. They had the characters of Chicken Tumor XVIII. The other fowls, kept long under observation, remained healthy.

*Chicken Tumor VII, an Osteochondrosarcoma. Filtration Experiment 2.*—Six normal fowls were injected in the pectoral muscle on the right side with 6 c.c. each of an active Berkefeld filtrate, in the right thigh muscles with 4 c.c. of the same filtrate to which had been added a little *Kieselguhr*. It has been repeatedly found that the thigh muscles furnish a relatively poor site of inoculation. Nevertheless, in four of the fowls rapidly growing cartilaginous tumors of multicentric origin appeared in the thigh (figures 3 to 8), whereas a pectoral tumor developed in only two of the four. It took the form of a discrete cartilaginous nodule in the track of the injecting needle. The other two fowls remained healthy. One of the tumor fowls furnished material for the experiment which follows and also for a successful attempt to dry the etiological agent. Some of the growths contained so much bone that the saw was required to lay them open.

*Filtration Experiment 4.*—Portions of a tumor extract prepared as usual and with the usual bacterial control were passed through one of the following Berkefeld cylinders: (a) and (b) two small N cylinders (No. 5), and (c) a medium sized V (No. 3). The tumor material came from one of the fowls of filtration experiment 2. Filtrates A and B were injected separately in amounts of 10 c.c. into the leg muscles of three normal fowls. Filtrate C with and without admixture of *Kieselguhr* was injected into the pectoral regions of the same fowls. Nineteen days later one had developed a tumor nodule 1.3 cm. in diameter at the spot where 5 c.c. of filtrate C with *Kieselguhr* had been injected. The nodule rapidly enlarged to a diffuse mass which led to the death of the fowl after six weeks in all. At this time a nodule had just appeared in the other pectoral region where 10 c.c. of filtrate C without admixture had been put. At autopsy another small discrete nodule was found in the left leg as the result of 10 c.c. of filtrate A without *Kieselguhr*. The other two fowls remained healthy.

*Chicken Tumor I, a Spindle-Celled Sarcoma. Injury Experiment 15.*—A active Berkefeld filtrate was prepared as usual and to half of it a little *Kieselguhr* was added. 9 c.c. of the mixture was injected into the pectoral muscles on one side of eight fowls, and on the other as control an equal amount of the plain filtrate. The results are shown in text-figure 1. It will be seen that when there was *Kieselguhr* tumors arose much more rapidly, and when palpable were already diffuse. In this instance nearly all the fowls responding with a tumor to the filtrate plus *Kieselguhr* eventually developed growths where the filtrate alone had been placed. Ordinarily this happens in only a small percentage.

#### SUMMARY ON FILTRATION.

The growths engendered by a Berkefeld filtrate have the distinctive characters of the strain of tumor which furnished the material for filtration (figures 1 to 9). In the case of Chicken Tumors I and VII growths so caused have themselves been successfully used for the preparation of filtrates, as for example in one of the experiments just cited. They have also been found to be transplantable. The results with Chicken Tumor XVIII are relatively meagre owing to the long period of latency after the injections,—more than five months in one case,—and to the slow growth of the filtrate tumors. The agents causing Chicken Tumors I and VII often pass the filter in large amount, as shown by the multicentric development of tumors in the region injected, but even when the element of tissue derangement is present, Chicken Tumor XVIII usually appears from one, or at most a few, centers. This is more probably due to low average virulence or resistance in the agent than to difficulties in filtration, for the findings show that all the agents pass or are held back by filters of about the same texture. They pass most V cylinders, many designated as N, but they are usually retained by the fine textured W cylinders. As might be expected from these results the agent of Chicken Tumor I,—the only one thus tested so far—fails to pass Chamberland bougies.

Not infrequently filtrates prepared from malignant material under the best conditions prove entirely innocuous. This is in most cases due to the narrow limits within which the agents are filterable. Two possibilities suggest themselves as accounting for these limits, first that the agents are formed bodies, second that if unformed they are associated with substances which clog the pores of the filters. Mucinous substances are so abundant in Chicken Tumor I

that coarse filters are soon completely stopped by very dilute extracts of the growth; but with VII and XVIII there is no such complication. Extracts of these tumors run rapidly through the filters and yet are often inactive. The agents then would seem to be of relatively large size among the filterable causes of disease.

The tumor resulting from a local injection of filtrate appears at the site of inoculation. As already mentioned the importance of tissue derangement for the action of all three causative agents is very great. The experiments show that when this factor is supplied by the addition of *Kieselguhr* to a filtrate the percentage of fowls that develop tumors is much increased and the growths themselves appear sooner and enlarge more rapidly (text-figure 1). The *Kieselguhr*, injected alone, does not cause tumors. A limpid filtrate injected in the breast muscle finds its point of action in the track of the injecting needle (figure 9), and there results a discrete growth from one center. Oftentimes, as ordinarily prepared, the filtrate contains a few particles from the interior of the filter, and the growth may then arise from several centers. But when powdered *Kieselguhr* has been added the growth is multicentric and appears all at once as a mass of coalescing foci. The microscopic findings show that the sarcomatous change in the reactive tissue about the *Kieselguhr* is not diffuse but punctate, and the growth of the little tumors is largely expansive (figure 10). In fowls inoculated intravenously with a filtrate of Chicken Tumor I the tumors have been found to arise at sites of tissue derangement.<sup>7</sup> Their incidence is approximately doubled when *Kieselguhr* has been introduced into the blood stream.

As already mentioned the reactive tissue called forth by *Kieselguhr* contains large numbers of giant cells. These are of ordinary foreign body type and enclose the *Kieselguhr* fragments. With the replacement of the reactive tissue by tumor the giant cells are destroyed (figure 10) and the *Kieselguhr* is set free. It can be found here and there among the sarcoma cells, which appear in no way affected by it.

<sup>7</sup> Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lviii, 1751.

*Causation of Chicken Tumors.*

I.31.12

Nº	FILTRATE + K.			FILTRATE ALONE			
	I/6	I/11	I/15	I/6	I/11	I/15	I/19
932	+	N	*	N	+	N	N
933	N	•	•	N	N	N	•
934	N	•	•	N	N	•	•
935	N	•	•	N	N	•	•
936	N	•	•	N	N	•	•
937	?	•	•	N	N	•	•
938	N	N	•	N	N	N	•
939	N	+	•	N	N	N	N

TEXT-FIG. I. CHICKEN TUMOR I. INJURY EXPERIMENT 15. Eight normal Plymouth Rock fowls, Nos. 932 to 939, were injected in one pectoral region with a Berkefeld filtrate of Chicken Tumor I, in the other with an equal quantity of the same filtrate to which had been added a little powdered diatomaceous earth (*Kieselguhr*). Examinations were made every week thereafter and the size of the tumors charted. It will be seen that where filtrate plus *Kieselguhr* was injected tumors appeared more frequently and much earlier, and were diffuse when first noted in sharp contrast to the discrete nodules on the other side.

## DESICCATION.

Since many microorganisms withstand drying and the tissue cells of the higher animals do not, workers have frequently used desiccation in attempts to separate an etiological agent from mammalian tumors. The literature need not be discussed since it deals only with negative results. The agents causing two of our chicken tumors, namely the pure spindle-celled sarcoma (Chicken Tumor I), and the osteochondrosarcoma (Chicken Tumor VII), resist drying. No special complications are met with in obtaining an active dry form of the agent of Chicken Tumor I, but in the case of Chicken Tumor VII successful results have been obtained only by the desiccation of frozen material. A few specimen protocols will be given.

*Chicken Tumor I. Desiccation Experiment 2.*—The tumor material, obtained from fowl 243 of the ninth transplantation generation, series A, was ground fine and spread thin in a desiccator containing sulphuric acid. A partial vacuum only was produced. After forty-eight hours the scales of dry tissue were pulverized in a mortar and replaced in the desiccator. After sixty-seven hours in all the powder was taken up in Ringer's solution by grinding, in the proportion of 2 gm. to 70 c.c. 5 c.c. of the turbid, viscous fluid were injected into each breast of seven normal hens. Six showed rapidly growing tumors at the end of the month. The seventh remained healthy. One of the fowls furnished tumor material for the experiment which follows.

*Desiccation Experiment 4.*—A fowl of experiment 2, with a large tumor, was killed and the neoplastic material dried as before, for forty-eight hours; ground; replaced in the desiccator for seventy-two hours more; and then sealed in glass and kept in the dark at about 2° C. Fifty-four days later 2 gm. of the material were taken up in 25 c.c. of Ringer's solution, and the thick suspension was inoculated in amounts of 5 c.c. into each breast of five normal hens. Three of these had developed tumors two weeks later, and one growth was already large. In a fourth fowl the tumor appeared later. The fifth remained quite healthy.

Some of the dried material was left sealed and in the cold for seven months. 3 gm. of it were then taken up in 20 c.c. of normal salt solution and 1 c.c. of the mixture was injected into one breast of eight fowls. Of these two had developed small tumors after forty days. The others remained healthy. The growths were typical Chicken Tumor I.

*Chicken Tumor VII. Desiccation Experiment 3.*—Fowl 549, carrying a large growth engendered by a filtrate (filtration experiment 2) was killed, and the tumor, which consisted of cartilage and precartilaginous, sarcomatous tissue, was ground fine and placed in a cold compartment at several degrees below 0° C., where in the course of forty-eight hours it gradually froze solid. While still frozen it was put in a cold desiccator over sulphuric acid, and the air exhausted until the manometer showed a pressure of less than 1 mm. of mercury. The desiccator was then placed immediately in an ice chest. At the end of three days the material was taken out and ground. It had the form of a light, brittle cake

of nearly the bulk of the fresh tissue, was cottony in texture, and ground with great difficulty. Twenty-four hours after grinding, 2 gm. of the powder were taken up in 25 c.c. of sterile water and of the thick fluid 5 c.c. were injected into the left breast of each of five normal fowls. Within a month one developed a growth measuring 4 by 9 cm. It was killed and portions of the tumor were used for the experiment that follows. The tumor consisted of a spindle-celled sarcomatous tissue undergoing cartilaginous differentiation in the way characteristic of Chicken Tumor VII. The other fowls, kept under observation several months, remained healthy.

*Desiccation Experiment 4.*—The material came from the susceptible fowl of the previous experiment. It was ground, frozen, and dried while still frozen, by the method just outlined. It remained in the freezing chamber three days, in the desiccator twenty-four hours, and was then powdered and placed in the ice chest for twenty-four hours more. Eight fowls were injected in the left breast with 5 c.c. each of a thick fluid made by taking up 2 gm. of the powder in 40 c.c. of distilled water. In the other breast glycerinized tumor tissue was placed at the same time (glycerin experiment 2). Within three weeks all except one of the injected fowls had developed a tumor mass at the site of inoculation of the dried material, the largest measuring 3.4 by 7.5 cm. Four of the fowls were allowed to die of their growth, the other three being killed and examined at various times. All the growths presented the character of Chicken Tumor VII, with much cartilage and in several instances bone. The eighth fowl remained healthy.

In this experiment there was the complicating factor of tumors developing synchronously from glycerinated material. But that the tumors which arose where dried material had been injected were engendered by this is certain, not only from the local character of the neoplastic disease in its early stages, but from many other instances that might be cited in which the dried tissue of Chicken Tumor VII has been successfully used to cause the growth.

#### SUMMARY ON DESICCATION.

The growths caused by dried tissue of Chicken Tumors I and VII appear at the site of injection and themselves furnish material which has a tumor-producing activity when dried. The etiological agent of Chicken Tumor I undergoes a gradual attenuation when the dried tissue is stored in the dark at a temperature slightly above 0° C., but after seven months is still capable of producing tumors. In the case of Chicken Tumor VII no attempt has been made to store dried material for longer than three weeks. At the end of this time it still produced tumors.

Numerous attempts have been made to obtain the agent of Chicken

Tumor XVIII in an active dry form, but as yet without success. In this connection it should be recalled that Chicken Tumor XVIII under the best conditions grows much more slowly than I and VII and that a filtrate of it gives rise only exceptionally to tumors and after a long latent period. The agents of Chicken Tumors I and VII when dried at low temperature are both very active; but the one easily survives drying at room temperature whereas the other fails to. Differences in the viability of organisms would well account for this.

The growths caused by dried material spring from multiple foci and are often diffuse when first palpable. The bits of dead tissue doubtless act to produce like *Kieselguhr* a tissue derangement favorable to the production of tumors.

#### GLYCERINATION.

The effect of glycerin has especial interest because of the ultra-microscopic organisms which retain their vitality when immersed in it. Loeb<sup>8</sup> has observed its effect upon mammalian tumor tissue. He placed pieces of rat sarcoma in pure glycerin, and, seventeen to twenty-four hours later, washed them in salt solution and injected bits into other rats. In some instances a tumor developed. If it can be assumed that the glycerin thoroughly penetrated the tumor tissue the finding is significant. For this reason we have repeated and enlarged upon Loeb's experiment, previous to observations with the chicken tumors. Briefly, the findings show that pieces of the Jensen rat sarcoma 0.5 cm. thick, when kept for twenty-four hours in pure glycerin at a temperature of 1° to 2° C., will sometimes give rise to tumors on being cut up and implanted; but the same material, finely chopped and stirred into the glycerin, yields only negative results, even when well washed and injected in quantity. Rat sarcoma treated according to the latter method occasionally retains its viability for twenty-four hours in a 12.5 or 25 per cent. glycerin mixture with Ringer's solution, but never in 50 per cent. glycerin, nor for a longer period (four days) in the weak dilutions mentioned. Hence it seems then that the positive results with pieces of rat tumor kept in concentrated glycerin are dependent on incomplete penetration.

<sup>8</sup> Loeb, L., *Virchows Arch. f. path. Anat.*, 1903, clxxii, 345.

Of the filterable entities that cause chicken tumors the two which withstand drying will also survive glycerination.

*Chicken Tumor I. Glycerin Experiment 3.*—13 gm. of fresh tumor tissue from fowl 582 (16th generation B) were ground with sterile sand, and suspended in 40 c.c. of Ringer's solution. The larger tissue particles were got rid of by straining, and the sand was allowed to settle out. Of the thick suspension of tissue 10 c.c. were stirred into an equal amount of pure glycerin, shaken until well mixed and placed in the ice box. After seven days the tissue fragments, which had by this time settled, were drawn off through the base of the tube by breaking a sterile glass projection in its wall. In this way all contamination with particles which had escaped the glycerin's action was avoided. The tissue suspension was now washed with Ringer's solution, centrifugalized, and of the pasty sediment 0.15 c.c. was injected into the right and left pectoral muscles respectively of three young Plymouth Rock fowls. Ten days later in one fowl a small sarcomatous nodule made its appearance at each injection site, and in a second after seventeen days a single nodule developed. The subsequent growth of the tumors was slow, but otherwise they proved characteristic of Chicken Tumor I. The third fowl remained healthy.

*Glycerin Experiment 2.*—Fresh tumor tissue was ground in a mortar with sand and taken up in Ringer's solution. By rapid centrifugalization the suspension was rid of all but extremely minute tumor fragments. To portions of it pure glycerin was added to the amount of 50, 25, and 12 per cent. respectively of the total bulk. The mixtures were placed in wide tubes and kept in the dark at 1° to 2° C. After nine days some of the material was withdrawn by breaking a projection at the base of the tube, as in the experiment already described. Twenty-two days later a fowl injected in each pectoral region with 0.4 c.c. of the mixture in 50 per cent. glycerin, diluted to 4 c.c. with Ringer's fluid, had developed very large tumors having the character of Chicken Tumor I. In the three fowls injected with 12 and 25 per cent. mixtures, variously diluted, growths had also appeared at this time.

Some tubes of the 12.5 and 25 per cent. mixtures were left untouched and in the cold for thirty-one days. Their contents were then drawn off as usual. One fowl injected in each breast with 3 c.c. of 25 per cent. mixture, undiluted, developed tumors. These first became palpable after six weeks. Another fowl injected in the same way with 1 c.c. of the 25 per cent. mixture made up to 2 c.c. with Ringer's fluid likewise slowly developed tumors. Three fowls inoculated with the 12.5 per cent. mixture, diluted with an equal bulk of Ringer's solution, remained healthy.

Higher percentages of glycerin have been used in the experiments with Chicken Tumor VII.

*Chicken Tumor VII. Glycerin Experiment 3.*—Cartilaginous tumor material was ground to a foamy pulp in a meat chopper and two mixtures were made with pure glycerin, in the amounts of (a) 2 c.c. tumor pulp and 8 c.c. glycerin, and (b) 4 c.c. tumor pulp and 10 c.c. glycerin. The actual quantities of tumor tissue were considerably less than those mentioned, as it had the form of a

soufflé. The mixtures were tubed and kept in the cold as usual. Sedimentation in the glycerin was extremely slow and shaking was done each day to keep the larger tissue fragments in suspension. After thirteen days the tubes were opened at the base, and the mixtures, made up to 20 c.c. with Ringer's solution, were injected in amounts of 5 c.c. into the right and left pectoral region, respectively, of three normal fowls. After two months one fowl developed a tumor that in another three months proved fatal. The growth was 6 cm. in diameter at this time and in addition to much cartilage contained bone. It had arisen at the site of injection of the mixture that contained the greater percentage of glycerin (8 c.c. to 2 c.c. of tumor). The other fowls remained healthy.

*Glycerin Experiment 4.*—Fresh cartilaginous tumor material was ground to a soufflé and three mixtures were made: (a) 10 c.c. of pure glycerin with an equal bulk of tumor material, (b) 10 c.c. of glycerin with 5 c.c. of tumor, and (c) 10 c.c. with 3 c.c. of tumor. These were kept in the ice box; shaken every day; at the end of ten days drawn off from below; and each made up to 20 c.c. with Ringer's solution and inoculated in amounts of 5 c.c. in one pectoral region of five normal fowls. Four of these died of intercurrent disease. Ninety-nine days after the injection the fifth had developed a tumor measuring 8 cm. at the inoculation site of mixture (c), and one of half this size where mixture (a) had been put. Both growths were characteristic of Chicken Tumor VII and both contained bone.

#### SUMMARY ON GLYCERINATION.

The experiments leave no doubt that the tumor tissue was thoroughly penetrated by glycerin. The agent of Chicken Tumor I retains some activity for at least seven days in 50 per cent. and for thirty-one days in 25 per cent. glycerin. The effect upon it of higher concentrations has not been tested. In the case of Chicken Tumor VII the amount of glycerin has ranged from 50 to about 90 per cent., but no attempt has been made to determine the period of survival of the agent. That it remains active in the high concentrations for at least thirteen days is shown by experiment 3. Glycerination undoubtedly has an attenuating action on both agents. The tumors develop in few hosts, and after a relatively long latent period, and often grow slowly. It has been repeatedly noted that the activity of the agents is best retained in high concentrations of glycerin. This might be thought due to differences in penetration of the tumor tissue attendant upon differences in concentration, were it not for the findings with rat sarcoma. Here concentrated glycerin proves the more injurious. Tissue autolysis in the dilute glycerin mixtures affords a better explanation of the results with the avian tumors. The most active agent, that of Chicken Tumor I,

has been found to be quickly destroyed by autolysis of the tumor tissue.

Repeated attempts to preserve in glycerin the agent causing Chicken Tumor XVIII have been unsuccessful.

#### SEPARATION OF THE AGENTS BY OTHER METHODS.

Only in the case of Chicken Tumor I have still other attempts been made to distinguish the tumor-producing agent from the neoplastic cells. Ultraviolet rays kill the cells and leave the agent unharmed.<sup>9</sup> The method requires careful control and is not available for the separation of large amounts of the agent. The resistance of the agent to heat is only very slightly greater than that of the cells.<sup>10</sup> On the other hand, the agent withstands freezing and thawing which reduce the associated tumor tissue to a pulp.

#### DISCUSSION.

In the first attempts to isolate a causative agent from Chicken Tumors VII and XVIII exact precautions were taken to avoid a possible contamination with the agent of Chicken Tumor I, though such contamination had never been observed in the many routine transplantations of these growths. To avoid exposure of the material to the laboratory air it was ground in a large sterile box and otherwise protected. The results of the experiments showed that these precautions were unnecessary, for the character of the tumors engendered by the agents effectually proved that they were not the result of contamination. Each agent produces only growths of the kind from which it came. One stimulates connective tissue to proliferate and elaborate cartilage, ultimately to be replaced in greater or less part by bone (Chicken Tumor VII); another causes connective tissue to proliferate and form large undifferentiated spindle-celled masses (Chicken Tumor I); while a third engenders, like the second, a spindle-celled growth, but one containing much collagen, and characteristically fissured by blood sinuses, into which the growth shows a tendency to extend, resulting in a complex intra-canicular pattern (Chicken Tumor XVIII). The behavior of the

<sup>9</sup> Rous, P., *Jour. Exper. Med.*, 1913, xviii, 416.

<sup>10</sup> Rous, P., and Murphy, J. B., *Jour. Am. Med. Assn.*, 1912, lviii, 1938.

several growths is as different as is their histology. Chicken Tumor I metastasizes to the lungs by preference, then to the further viscera; Chicken Tumor VII almost never gives metastases; and Chicken Tumor XVIII frequently disseminates to the muscles without other secondary localization. The individuality of the agents as exemplified in the neoplasms that they cause is not altered by attenuating them, yet it should be mentioned that Chicken Tumor XVIII, the fissured sarcoma, has recently shown a tendency, like some complex mouse tumors, to lose with repeated transplantation its histological peculiarities and become an undifferentiated spindle-celled sarcoma. Ultimately the agent causing it may produce tumors not very different from Chicken Tumor I.

The tumors engendered by the filterable agents become palpable only after a latent period,—which in the case of Chicken Tumor XVIII is usually several months. Chicken Tumors I and VII appear more promptly. Fowls which fail to develop the growths within one month after an injection of the agent remain free of them, as a rule. When large amounts of filtrate plus *Kieselguhr*, or of dried or glycerinated material, have been injected the resulting growths are diffuse when first observed, owing to proliferation from many foci, and they quickly become massive; but the period of latency—at least eight to ten days in the case of Chicken Tumors I and VII—is not appreciably shortened. That the greater part of this period is one of actual latency with tumor cells absent as yet, and is not merely an interval during which the tumor is growing but clinically imperceptible, has been determined by the early microscopic examination of sites where the agent is known to be present and active.

The injection of large amounts of the agent of Chicken Tumor VII results in tumors that grow progressively and soon lead to the death of the fowl; whereas the growths developing after implantation of a small bit of the tumor enlarge slowly and in most fowls become stationary and eventually retrogress. This difference might be thought due to the influence of dosage as affecting resistance,—a factor of much importance in the case of mouse tumors,—or it might conceivably result from the circumstance that growths induced by an agent are elaborated by the host's own tissues, while

such as result from transplantation represent tissue growing in a host to which it is strange. That the latter explanation is not fanciful has been shown by experiments with Chicken Tumor I demonstrating the existence in fowls of two types of resistance directed, the one against the tumor-producing agent, the other against the transplanted tumor cells.<sup>11</sup> But in the case now being considered differences in dosage as affecting resistance are probably at the root of the matter. For by the injection of large amounts of the fresh tissue of Chicken Tumor VII in the form of a pulp, progressively growing tumors, such as result from massive doses of the agent, can be obtained.

The growths resulting from injection of a tumor-producing agent into the skeletal muscles are at first purely local in character, even when a filtrate is employed. In only one among many autopsies have growths been found which by their situation suggested a possible primary dissemination by the blood stream. This case is cited in one of the protocols (Chicken Tumor XVIII, filtration experiment 2). There is, of course, no reason why part of a filtrate, injected with a sharp needle, should not often pass directly into some blood vessel. But it has been shown with Chicken Tumor I that the direct intravenous inoculation of an active filtrate usually fails to produce tumors.<sup>12</sup>

Many fowls are resistant to the tumor-producing agents in any of the forms that we have used. Glycerination markedly reduces the agents' activity and desiccation does so to a less degree, and roughly in proportion to the length of time that the material is kept after drying. Both filtrates and dried tissue prepared under the best conditions from malignant material are sometimes unaccountably inactive. More often the inactivity can be traced to the use of too concentrated extracts for filtration, too finely textured a filter, or too slow a process of drying.

The findings with the three tumor-producing agents have a striking similarity and it is difficult to avoid the conclusion that the three are of one class, whatever that class may be. All give rise to dis-

<sup>11</sup> Rous, P., *Jour. Exper. Med.*, 1913, xviii, 416.

<sup>12</sup> Rous, P., Murphy, J. B., and Tytler, W. H., *Jour. Am. Med. Assn.*, 1912, lviii, 1751.

eases of neoplastic character, all act after a more or less pronounced latent period, the action of all depends to a striking extent on associated tissue derangement, and all pass through Berkefeld cylinders of about the same porosity, being held back by others of slightly finer grade. Two of them resist drying and can be preserved in glycerin. The third, which fails to retain its activity when so treated, causes tumors that are of relatively very slow growth (Chicken Tumor XVIII). Since in our experience the separation of a tumor-producing agent is largely a question of the growth's malignancy, it seems not improbable that with selective passage of the neoplasm an agent may eventually be obtained from it that is resistant to drying and glycerination.

The separation of etiological agents from three chicken tumors of such diverse character as those we have employed is strong evidence for the view that many other growths of the fowl have a like cause. It is hardly necessary to point out that were the latent period of Chicken Tumor XVIII, when produced by the specific agent, somewhat longer than the two to six months observed in the present investigation, or were the agent only very slightly more difficult to separate from the tissue by filtration, its presence would not have been demonstrated. Chicken Tumor XVIII would then have remained, with the sarcomata of the rat and mouse, among the transplantable tumors without a cause separable from tissue cells.

#### CONCLUSION.

A causative agent has been separated from three chicken tumors of very different sort, namely a spindle-celled sarcoma, an osteochondrosarcoma, and a spindle-celled sarcoma peculiarly fissured by blood sinuses. This was accomplished after the tumors had been transplanted repeatedly and their malignancy enhanced. Each of the tumor-producing agents is a distinct entity in that it gives rise only to growths of the precise kind from which it has been derived. All pass through Berkefeld cylinders impermeable at the same test to small bacteria, and two of the three retain their activity in tumor tissue that has been dried or glycerinated. All are strikingly dependent for their action on derangement of the tissue with which they are brought in contact. The general findings strongly

suggest that the agents are of about the same size, and of the same natural class. It is perhaps not too much to say that their recognition points to the existence of a new group of entities which cause in chickens neoplasms of diverse character.

#### EXPLANATION OF PLATES.<sup>18</sup>

##### PLATE 7.

FIG. 1. Tumors caused by a Berkefeld filtrate of an extract of Chicken Tumor XVIII (a spindle-celled sarcoma fissured by blood sinuses) in Ringer's solution (filtration experiment 2). The sternum has been cut away and the body of the fowl eviscerated. In the left pectoral muscles is the large, pale, primary growth. On both sides at the junction of the sternal and vertebral ribs is the neoplastic rosary described in the text. That on the right has been cut through vertically. Its individual nodules have coalesced.

##### PLATE 8.

FIG. 2. A section of one of the growths produced by a filtrate of Chicken Tumor XVIII (filtration experiment 2). The fissuring with blood channels, sometimes accompanied by intracanalicular growth, is characteristic of this tumor. The numerous black points in the channels are the nuclei of the red blood corpuscles.

FIG. 3. Large osteochondrosarcoma produced by the intramuscular injection of 4 c.c. of the Berkefeld filtrate of an extract of Chicken Tumor VII (filtration experiment 2). The fowl was killed when comatose, eighty-seven days after the injection. Its emaciation should be noted.

##### PLATE 9.

FIG. 4. The growth shown in the preceding photograph, after it had been sawed open. Scattered amid the smooth, whitish cartilage is much bone with red marrow.

FIG. 5. An early stage of an osteochondrosarcoma produced by a filtrate of Chicken Tumor VII (filtration experiment 2). The fowl was killed when the tumor was first noted, eighteen days after injection. Here and there in the pre-cartilaginous tissue, which has the general character of a spindle-celled sarcoma, the matrix of cartilage is in process of formation.

##### PLATE 10.

FIG. 6. Another portion of the growth illustrated in figure 5. The formation of cartilage is well advanced.

FIG. 7. A section of the growth shown in figures 3 and 4. The cartilage is in process of replacement by bone. Note the calcification and the abundant red bone marrow.

<sup>18</sup> All the microscopic sections were stained with methylene-blue and eosin.



FIG. 1.

(Rous and Murphy: Causation of Chicken Tumors.)



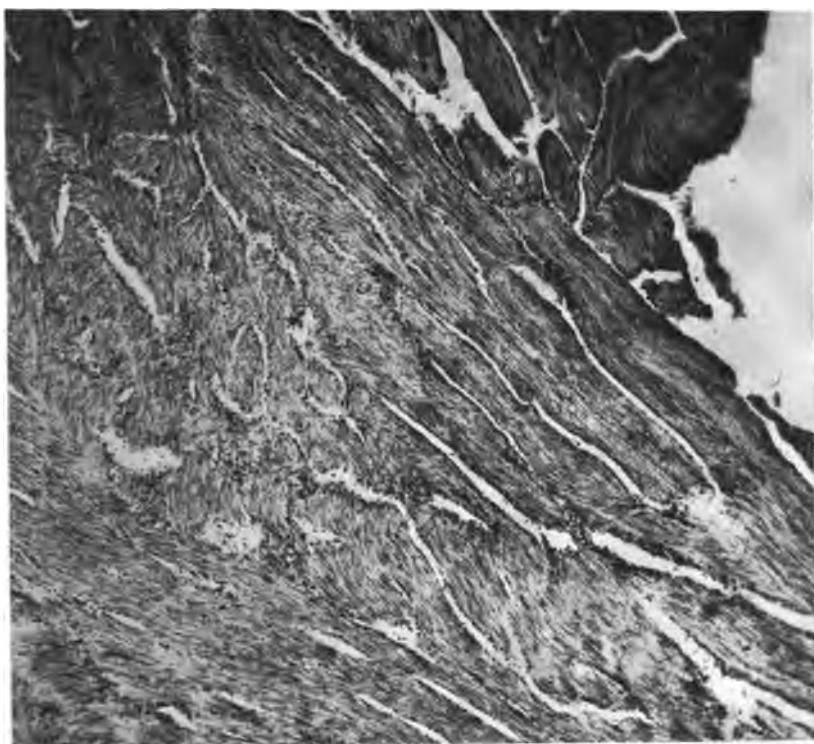


FIG. 2.

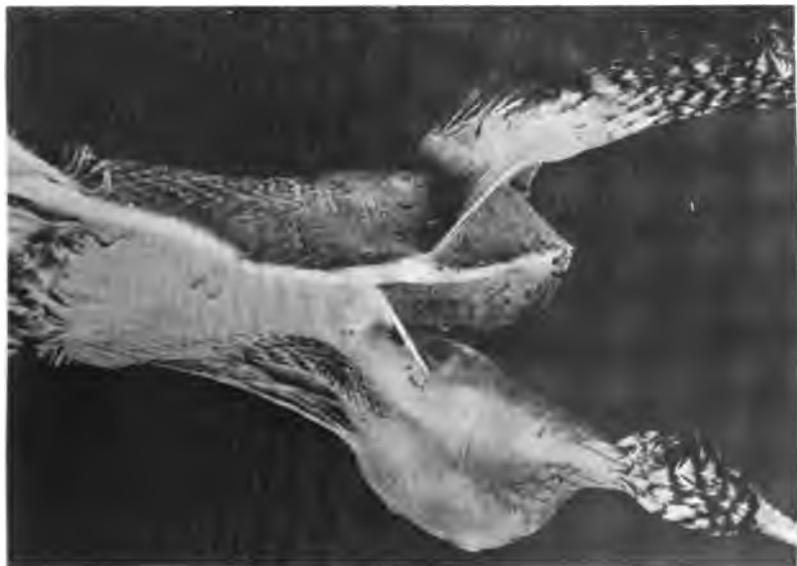


FIG. 3.

(Rous and Murphy: Causation of Chicken Tumors.





FIG. 4.

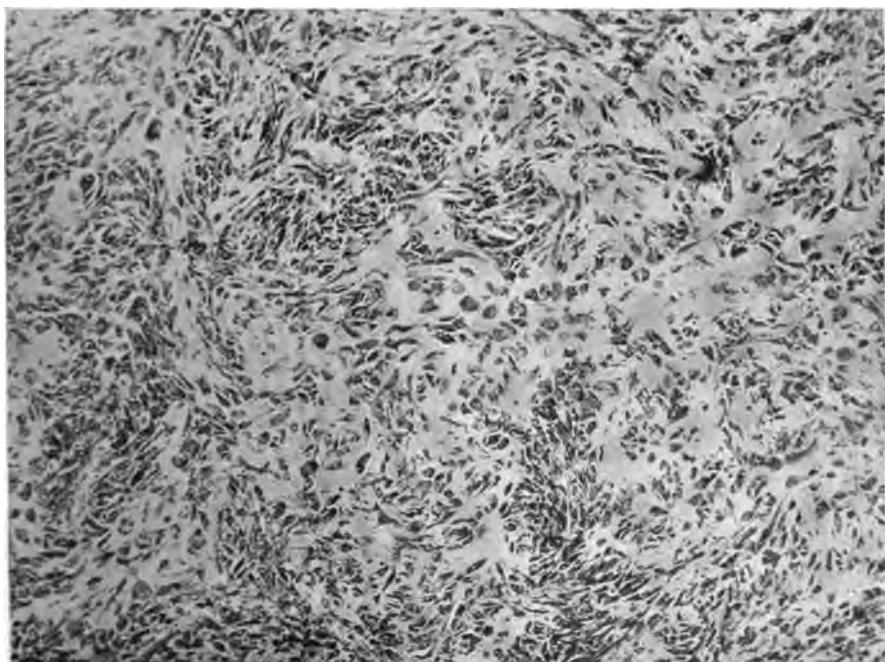


FIG. 5.

(Rous and Murphy: Causation of Chicken Tumors.)



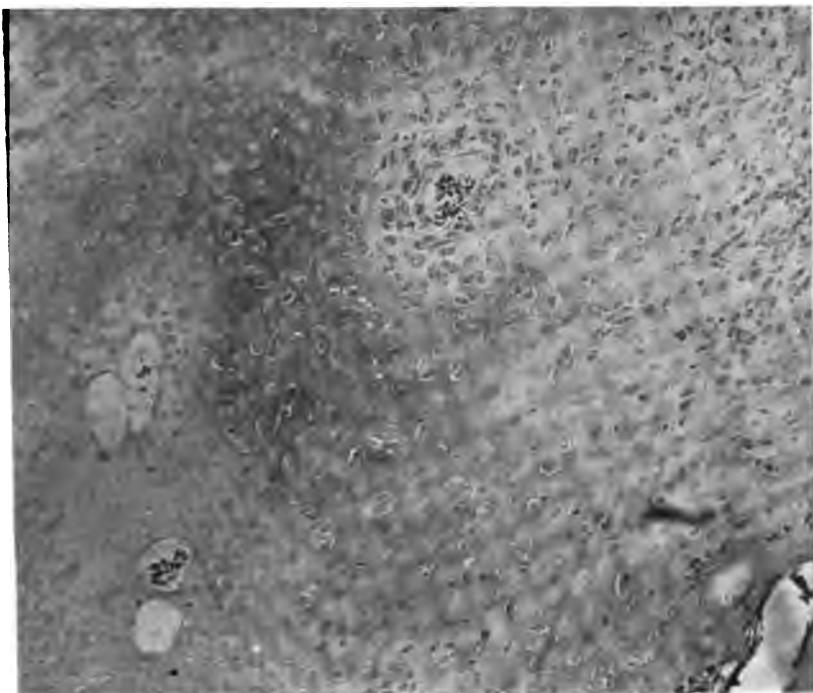


FIG. 6.

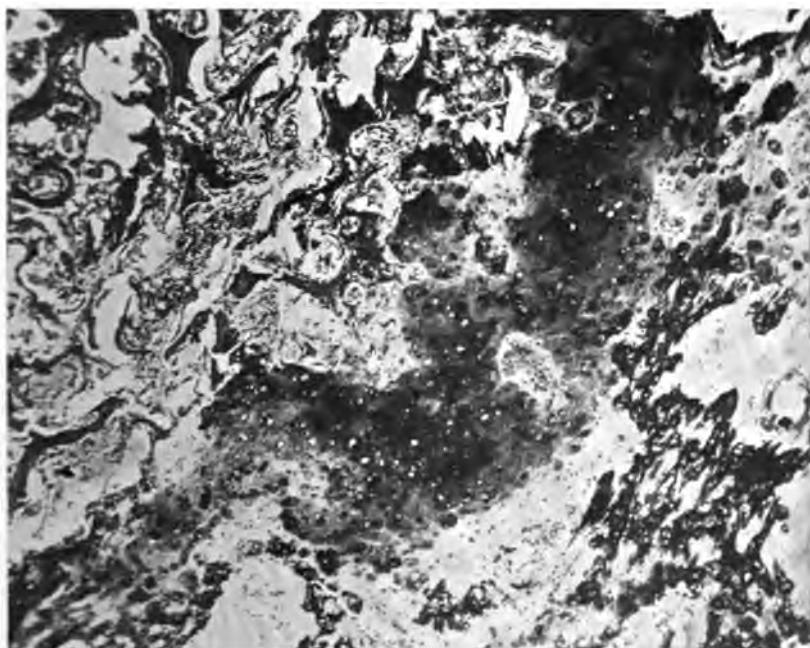
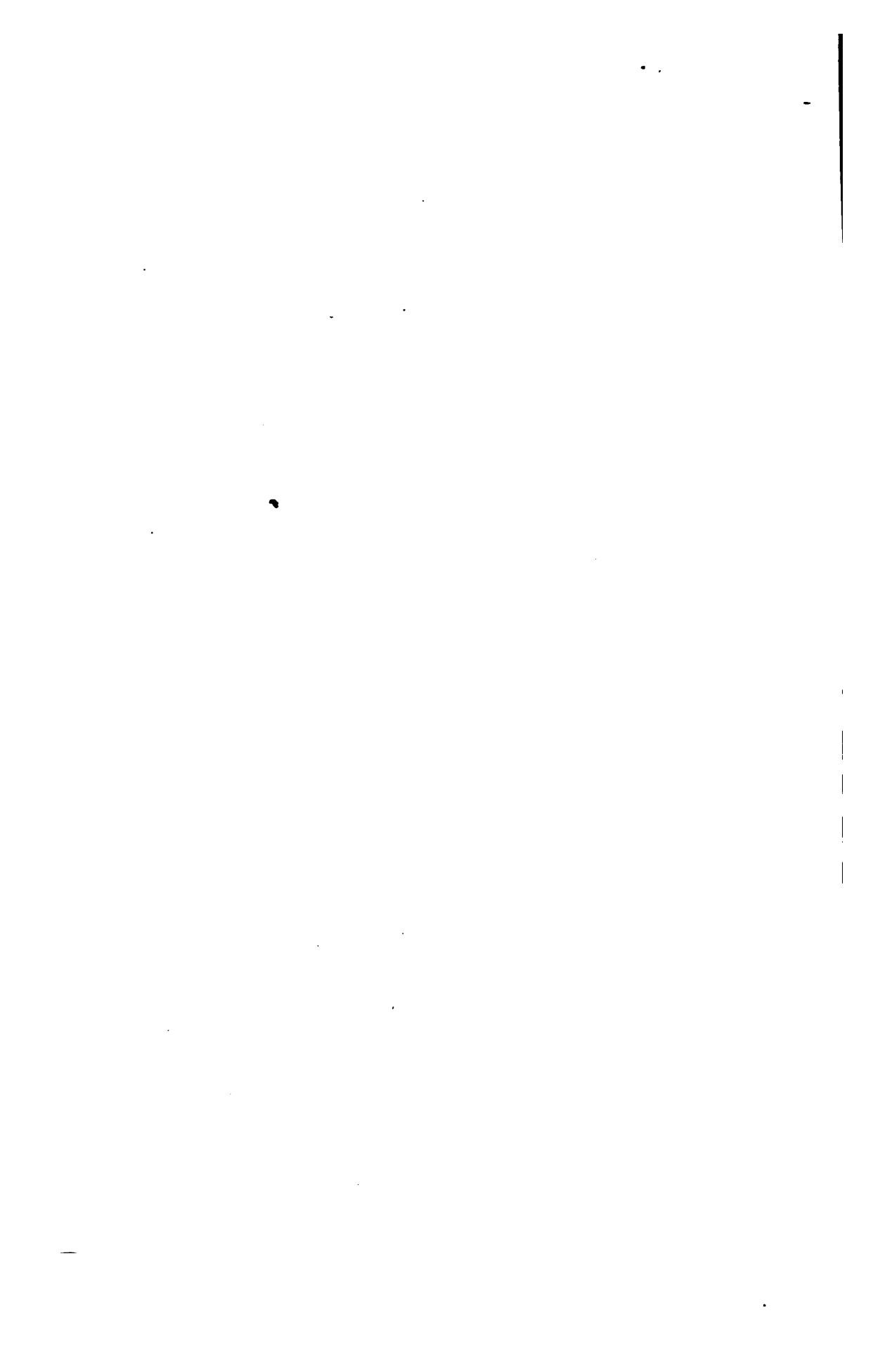


FIG. 7.

(Rous and Murphy: Causation of Chicken Tumors.)



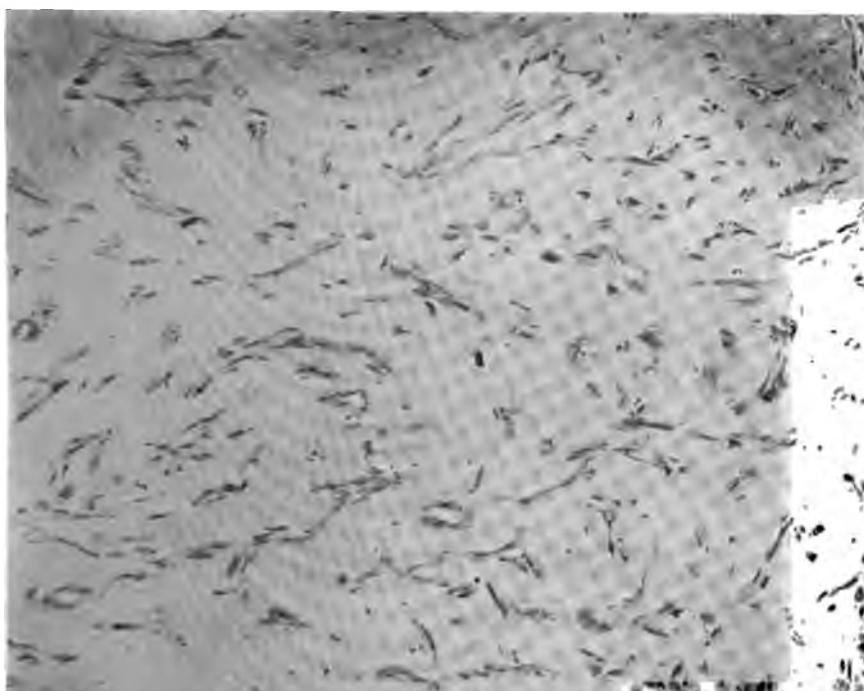


FIG. 8.

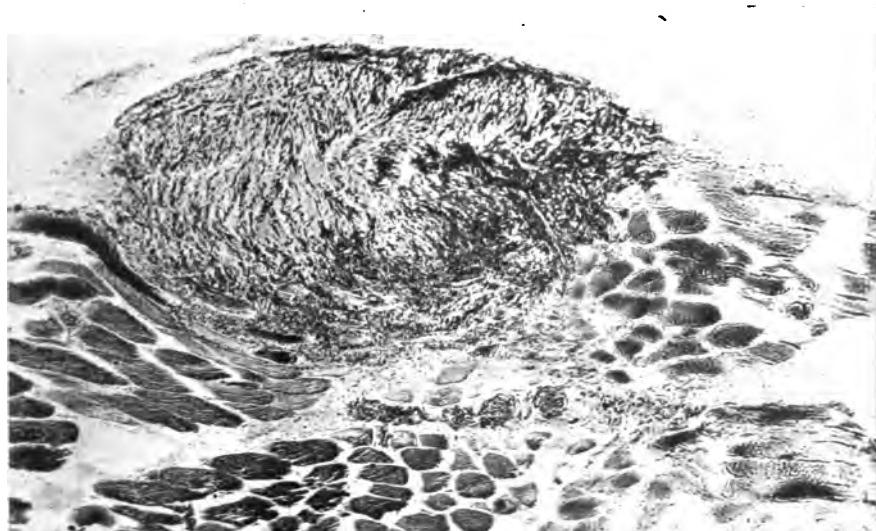


FIG. 9.

(Rous and Murphy: Causation of Chicken Tumors.)



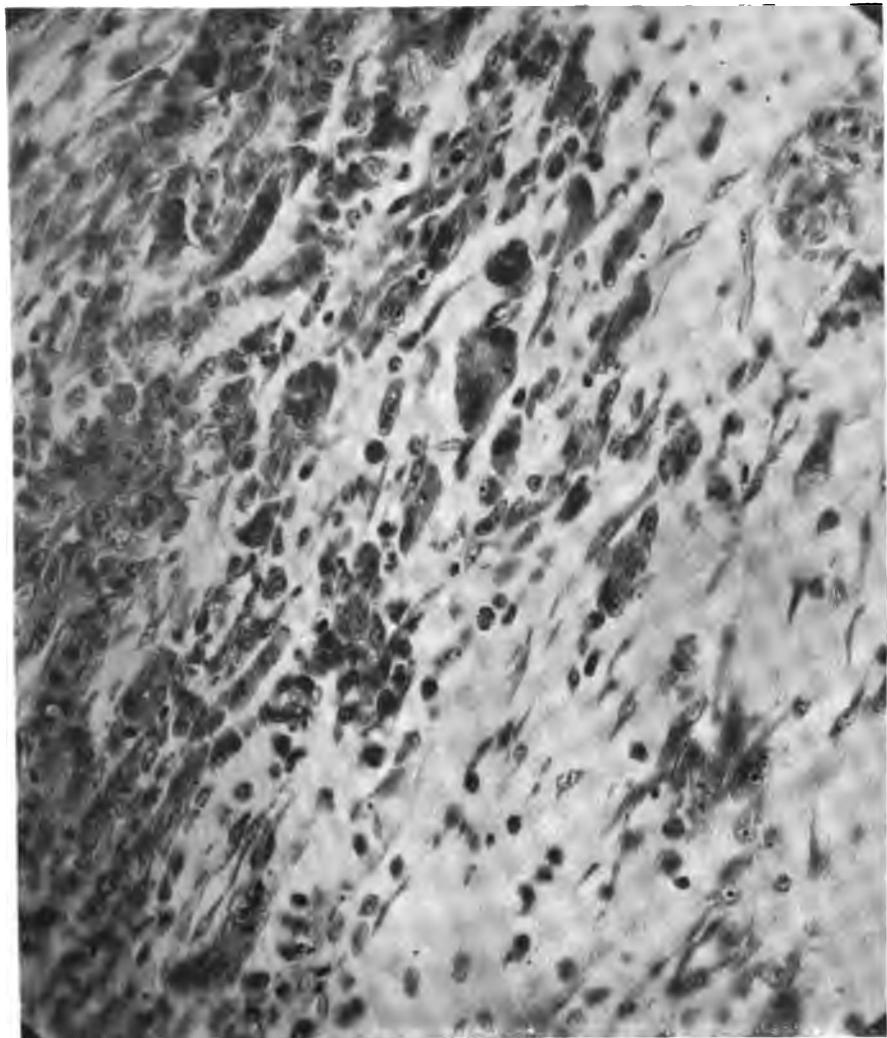


FIG. 10,

(Rous and Murphy: Causation of Chicken Tumors.)



PLATE II.

FIG. 8. Another portion of the same growth. An unusual intermediate stage in the formation of cartilage.

FIG. 9. A spindle-celled sarcoma developing after the injection of 0.5 c.c. of a filtrate of Chicken Tumor I. No *Kieselguhr* had been added to the filtrate. The growth arose in the sheath of the outermost pectoral muscle, where the needle had been thrust through, and it was excised eleven days after the injection, at which time it measured only 0.15 cm. in diameter. There was no recurrence. The tumor is distinctly sarcomatous, sharply localized, and is enlarging to a considerable extent by expansive growth, as shown by the way in which the muscle fibers are pressed to one side. Here and there it has begun to infiltrate. About it there is a slight round-celled reaction.

PLATE 12.

FIG. 10. The border of an osteochondrosarcoma that developed after the injection of a filtrate, on the basis of a *Kieselguhr* reaction. The fowl was killed while the tumor was yet very small and before cartilage had been laid down in it. The reactive tissue has been compressed into strata by the expansive growth of the tumor. The tumor cells, which are of fibroblastic character and occupy the upper half of the picture, are invading and replacing the giant-celled, reactive tissue about the *Kieselguhr*. Numerous fragments of this latter can be seen. With the destruction of the giant cells the fragments are set free within the tumor. Here they induce no evident reaction.

## FURTHER OBSERVATIONS AND EXPERIMENTS ON GOITRE (SO CALLED THYROID CARCINOMA) IN BROOK TROUT (*SALVELINUS FONTINALIS*).

### III. ITS PREVENTION AND CURE.\*

By DAVID MARINE, M.D.

(*From the H. K. Cushing Laboratory of Experimental Medicine of Western Reserve University, Cleveland.*)

#### PLATES 13 TO 17.

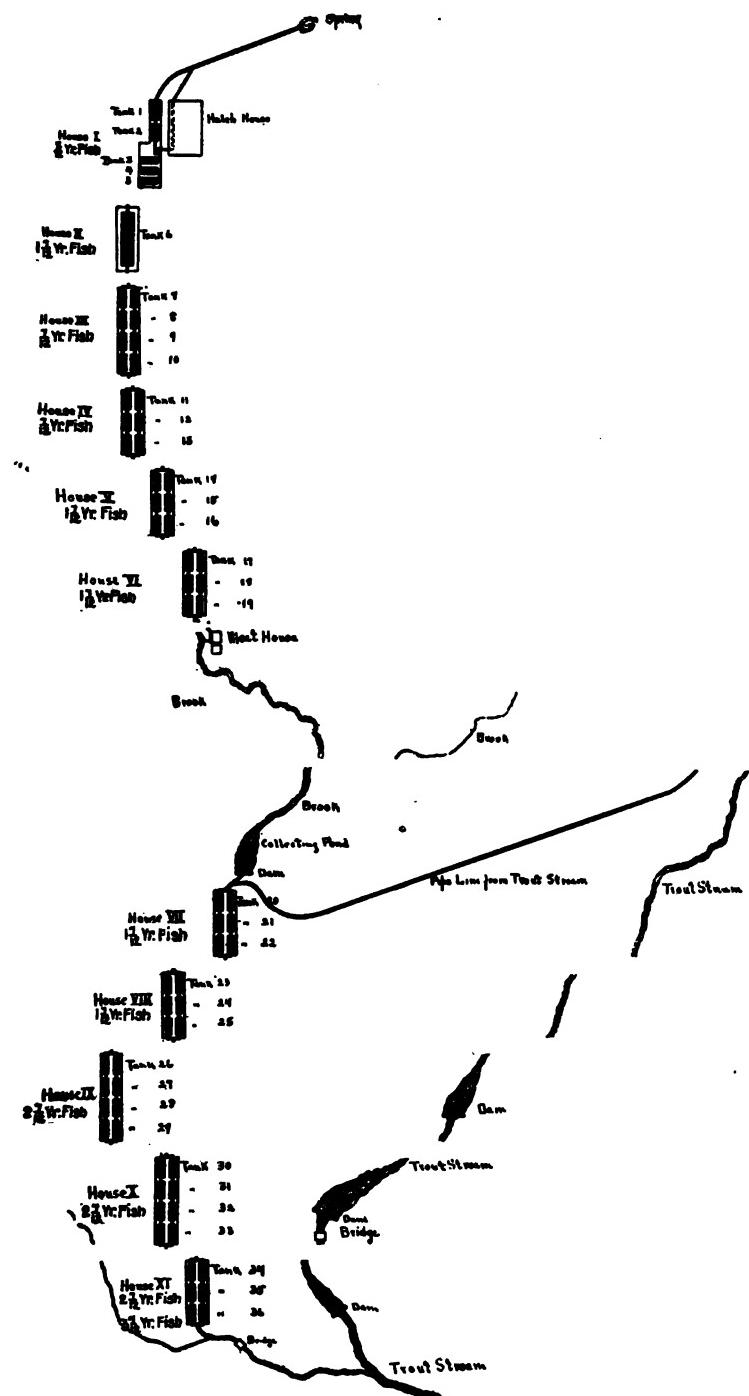
Investigations (1) made at the trout hatchery of the Blooming Grove Hunting and Fishing Club during 1909 and 1910 showed that all the fish were then goitrous. It was found that the thyroid hyperplasia began in the fry as soon as feeding was instituted, and advanced overgrowth was present at the fourth month of extra-oval life; that the overgrowth progressively increased to the stage of clinical detectability, as ascertained by the reddening of the pharyngeal floor over the thyroid area, about the tenth month in this hatchery; that visible goitres usually manifested themselves about the beginning of the second year, though they may be present as early as the sixth month, depending on the favorableness of conditions for overgrowth, and progressively increased during the second and third years; that older fish were more resistant and tended toward spontaneous recovery; that the water in which these fish lived was not naturally goitre-producing, since fish living wild in the stream and raceways did not develop thyroid overgrowth.

It was further shown that overcrowding and overfeeding with the highly abnormal and incomplete diet of hog's liver and heart were the major gross etiological factors, and of these the food was the more important factor in bringing about a fault of nutrition which stimulated the thyroid to compensatory overgrowth. No evidence was obtained that the disease was either infectious or contagious, or that a direct *contagium vivum* could account for the phenomenon.

The above summary of the work of 1909 and 1910 will serve as an introduction to the observations included in this report, which were made during August, 1913, and deal with the effect of changing the food from liver to fresh sea fish.

• *Comparison of the General Conditions Prevailing in 1909, 1910, and 1913.*—The water supply has remained unchanged as regards source and volume (text-figure 1). The Club is holding more trout in each pond than in 1909 and 1910, hence the untoward factor of

\* Received for publication, October 9, 1913.



TEXT-FIG. 1. Semidiagrammatic drawing of the plan, arrangements, and location of the hatchery, water supplies, houses, and retaining tanks.

overcrowding is increased. The distribution of the fish is in general the same (text-figure 1),—the fry<sup>1</sup> (hatch of 1913) in the upper, the nineteen months old trout in the middle, and the thirty-one months old trout in the lower ponds. The fry have always been scrupulously cared for by daily cleaning and sweeping the troughs and ponds. With the older fish (nineteen and thirty-one months) there is clearly less detritus in the ponds than in 1909 and 1910, although little additional attention has been paid to these ponds, the difference being due mainly to the change of food. Therefore, apart from the increased number of fish being held in the same pond space and the water supply, the only important change has been in the food.

*The Food.*—Beginning with October, 1911, fresh sea fish (butter fish (*Stromateus triacanthus*) and occasionally weak fish (*Cynoscion regalis*)) have been fed six times weekly to the older fish to the exclusion of all other foods. The fry are fed two to three times daily as in previous years, with finely divided hog's liver for the first four to five months of life, then heart muscle (hog) is added to this diet. This is continued until October (*i. e.*, nine months), when all the fry are removed to the ponds. At this time the change to fish diet is also made and maintained during the remainder of their stay in captivity.<sup>1</sup>

<sup>1</sup> It is as yet impossible to change to the fish diet earlier than the ninth month on account of the inability to hash the fish finely enough with the present machinery. This will soon be remedied, however, and then much of the thyroid overgrowth that now occurs during the period of liver feeding will be prevented. Several kinds of fish have been tried as food, among which are sea bass (*Centropristes striatus*), herring (*Clupea harengus*), weak fish (*Cynoscion regalis*), and whiting (*Merlangus americanus*). All have been found unsuitable either on account of bones or scales and have been abandoned. The fish scales being unaffected by digestion form rouleaux in the gut and produce intestinal obstruction followed by the usual sequelæ of enteritis, distention peritonitis, and death. I had the opportunity of examining two mild cases of partial intestinal obstruction, although for the past year only an occasional large scaled fish has been included in the food. In the two fish examined the obstruction was intermittent, as the scales were small (from weak fish) and could only obstruct when arranged transversely in the gut. There was well developed enteritis of the straight gut with extension through to the mesentery. Bones, if fine and rigid as in herring, are not broken up by the hasher and often perforate the stomach wall before the gastric acid can decalcify them. Fish with cartilagenous endoskeletons, such as skates, rays, sharks, etc., have not been tried owing to the difficulty as yet in

*Comparison of the Gross Appearance of the Fish of 1913 with Those of 1909 and 1910.*—Very striking differences are apparent. The nineteen months and the thirty-one months old fish are active and alert, while those of corresponding ages in 1909 and 1910 were sluggish and lumbersome. In handling large numbers of them in each of these three years, the 1913 fish are clearly stronger, show more fight, fatigue less easily, and do not die so quickly when removed from the water.

The fish are now more trout-shaped, that is, they have lost the pot-bellied appearance of 1909–1910. The skin pigments are brighter and better differentiated. It will be recalled from the descriptions of 1909–1910 that in all the fish the black pigment was excessively developed. The greenish grey marbling of the back was absent and the orange and pink color spots of the sides were wholly invisible. So also the orange and yellow colors of the fins and ventral surface were nearly absent. The silvery sheen of the sides was masked by the black pigment. In the fish of 1913 the colors approach closely those of the normal trout. The greenish marbling of the dorsum is detectable. The lateral color spots are well differentiated and the yellow and orange pigment of the ventral surface and fins are developed. The silvery sheen of the sides is perhaps better developed than in the average wild trout.<sup>2</sup>

As regards weight I have no exact data, although the impression is distinct that they are a trifle smaller on the average than in 1909 and 1910. The fry of 1913 are to all appearances the same as on previous examinations.

obtaining them in the markets. There seems no objection to their use since they would obviate the two great objections, scales and sharp perforating bones, and in addition might be less expensive than the usual market fish. Butter fish (*Stromateus triacanthus*) have been found least objectionable of the obtainable fish and are now used almost exclusively. They are received frozen and therefore in better condition than usually obtains with liver.

<sup>2</sup> These colors being readily adaptable to environment are modified by the color of the water, hence native trout from the brook are slightly darker than trout living in the spring water of the hatchery. It may also be recalled that in the experiments of 1910 the excessive black pigment diminished rapidly upon changing the food and removal of the fish from the ponds to troughs or to the trout stream. It would therefore appear that the food was a factor in the excess of the black pigment developed.

Of other diseases independent of goitre, as the myxosporidian infection (Taumelkrankheit, cramps), fungus, and enlarged gills (so called sore gills), there is a definite decrease.

*Comparison of the Gross Thyroid Condition of 1909 and 1910 with That Obtaining in 1913.*—These data are obtained from the clinical examination of the thyroid areas of a series of fish from the ponds for the presence of (a) reddening of the pharyngeal mucosa between the first and third gill segments as the first clinical evidence of thyroid overgrowth, and for (b) visible goitres projecting either dorsally in the floor of the mouth or in the gills, or ventrally in the angle of the attachment of the operculum as a late sign. The fry were not thus examined.

TABLE I.

Pond No.	October 12, 1909.				June 27, 1910.				August 10, 1913.			
	Age in yrs.	No. of fish examined.	Reddening of pharyngeal mucosa.	No. with goitre.	Age in yrs.	No. of fish examined.	Reddening of pharyngeal mucosa.	No. with goitre.	Age in yrs.	No. of fish examined.	Yellowish discoloration of pharynx.	No. with goitre.
6					1.5	41	10	0	1.7	25	1? trace	0
14					1.5	51	45	1	1.7	32	2? trace	0
19	1.8	210	197	7	1.5	60	51	2	1.7	22	2 trace	0
20	1.8	210	175	3	1.5	54	40	0	1.7	30	0	0
21									1.7	25	0	0
25	1.8	67	61	2	1.5	48	41	1	1.7	24	1 trace	0
26									2.7	22	3 trace	0
32	2.8	60	58	2	2.5	50	38	1	2.7	29	3 trace	0
35									2.7	32	4 trace	0
36	1.8	210	183	4					3.7	16	3	0
Totals		757	89%	0.23%		344	74%	0.17%		257	0.07%	0.0%

From the above tabulation it is seen that in 1909 89 per cent. of the fish had distinct reddening of the pharyngeal floor, and 0.23 per cent. had visible goitres. In 1910 a similar examination of fish from the same ponds and of nearly the same ages showed distinct reddening of the pharyngeal floor in 74 per cent. and visible goitres in 0.17 per cent. In 1913 a third examination of the fish from the same ponds and of approximately the same ages as those of 1909 and 1910 showed only a slight yellowish discoloration of the pharyngeal floor<sup>3</sup> in 0.07 per cent., and visible goitres in none.

<sup>3</sup> The reddening of the pharyngeal floor is due to the vascular thyroid tissue growing up to the pharyngeal mucosa. The yellow discoloration of the same region is due to the thyroid follicles rich in colloid and decreased vascularity, i. e., recovery or colloid stage of a preceding hyperplasia.

These percentages illustrate the average condition of all the fish at the time of the examinations and are controlled by the histological examination of specimens removed at the same time from all the ponds during each of these years. The differences in the gross condition of the thyroids for the years 1909 and 1910 are unimportant but illustrate the severity and extent of the disease. The differences in the thyroid conditions of 1909-1910 and of 1913 were beyond what I had expected and demonstrate clearly that the disease in the 1913 fish had been completely arrested. There were none with reddening of the floor of the pharynx nor with goitre, hence none with any active overgrowth of the thyroid. The few instances where slight yellowish discoloration was present is indicative of colloid-filled follicles beneath the mucosa. How can this most striking difference in the thyroid condition be accounted for? The only noteworthy determinable factor of difference between the conditions prevailing in 1909 and 1910 and those of 1913 is the food,—sea fish in 1913 and liver in 1909 and 1910. That the change of food accounts for the differences in the thyroid condition will become more apparent from the following observations and comparisons of the histological conditions of the thyroids of the entire pond series for the three years.

*Observations on the Histological Condition of the Thyroids from Specimens from All Ponds.*—(Figures 1, 2, and 3, and table II.) For comparison I have tabulated the anatomical state of the thyroids in 1909 and 1910 with that of 1913. In this table all the fish are included: (1) the fry, (2) the one to two years old, and (3) the two to three years old fish.

In the case of the fry for 1909-1913, all conditions, including food, water, crowding, cleaning, and general hygienic conditions are as nearly identical as it is possible to keep them. This is most fortunate in that it makes comparisons of the thyroid conditions for each year possible under constant conditions. It also gives a broad foundation for estimating the average thyroid hyperplasia present in the fry and is therefore valuable for checking any changes found in the older fish. With the one to two years old trout and the two to three years old trout of 1913 all conditions except the food are similar to those of 1909 and 1910. The one to two years old trout of 1913 have been fed sea fish for the past ten months. The two to three years old trout have been fed sea fish for twenty-two months, while the one to two years and two to three years old trout of 1909 and 1910 had been fed with liver only.

All the fry of 1909, 1910, and 1913 exhibited the same general thyroid condition of active hyperplasia (figures 4, 5, and 6). The thyroid area is completely filled with thyroid tissue and extension to the adjacent bone and muscle is present

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in all. The differences in the different years are only of slight degree and are probably dependent on the slight age differences at the time of examination: 1909, 8 to 9 months old; 1910, 6 months old; 1913, 7 months old. The individual variations within a given year are also slight and in general the same for all years. The examinations in each year comprised two specimens taken without choice from the twenty-one troughs in the hatch house and thirteen pairs of ponds of the pond series for the fry and also two specimens for each pond of the remaining twenty-three pairs for the older fish. From the examination of the fry in 1910, which, in addition to the above, included a series taken at weekly intervals from the time of hatching (January 15) to October, it was shown that the thyroid overgrowth began as soon as the fish began feeding and was easily detectable at the fourth month of extra-oval life, whence it progressively increased through the following months of the first and second years, rarely becoming clinically detectable before the tenth month at this hatchery. In some hatcheries the over-growth has been clinically detectable at the sixth to the seventh month, depending on more favorable conditions for thyroid overgrowth. The fry of 1911 and 1912 were not examined, but these same fish were included in the 1913 examination as thirty-one months and nineteen months old fish, respectively, and from this examination there is complete evidence that their thyroids had undergone changes identical with those in the years 1909, 1910, and 1913. Passing now to the nineteen months old fish, one finds a most striking change also noted in the gross examination of the thyroid area. All the follicles are in the colloid state, although their distribution extends into the bone and muscle and often up to the pharyngeal mucosa. The fish from the same ponds and of approximately the same age in 1909 and 1910 all had well marked active hyperplasia (figures 7 and 8) greater in amount and more widely distributed in muscle, bone, gills, and pharyngeal mucosa than those of 1913. The growth in nineteen months old fish of 1913 (compare figures 9 and 10 with figures 11 and 12) was found to be completely arrested, and the hyperplasia had completely involuted, while in the fish of 1909 and 1910 of the same age the growth was extending rapidly. As the arrest of the growth corresponds in time with the change in food, and as no other factor of difference is present, one is forced to the conclusion that the food is the major cause of the change. While the fish have grown rapidly during the ten months since the feeding of fish began, their thyroid tissue is no greater in amount nor more extensive in distribution than that reached during the first ten months of life. So also with the thirty-one months old fish which have been fed fish for twenty-two months the thyroids are completely involuted to the colloid state and the amount and distribution of the follicles are the same as those of the nineteen months old fish, and hence no greater than that attained during the first ten months of life, although the fish have made their normal annual growth. One sees, therefore, fish nineteen months and thirty-one months old with thyroids identical in all particulars and no greater in amount nor more extensive in distribution than those of liver-fed fry of ten months in this hatchery. That the growth was arrested and involution started at the time of the change from liver to sea fish cannot be doubted, although for the sake of completeness it will be necessary to make a series of weekly examinations beginning before and continuing for some months after the change of food to fresh sea fish. This is now being done. Additional proof that change

TABLE II.

Pond or trough No.	Age at start of experiment	1909.		1910.		1913.		
		Extent of overgrowth.	Condition of thyroid.	Extent of overgrowth.	Condition of thyroid.	Extent of overgrowth.	Condition of thyroid.	
Trough 1	8	All subpharyngeal structures,—bone and muscle	Marked hyperplasia	6	Entire aortic area; no invasion of bone or muscle	Moderate hyperplasia	7	Entire aortic area; Marked invasion of bone and muscle
Trough 5	8	All subpharyngeal structures,—bone and muscle	Marked hyperplasia	6	Entire aortic area; no invasion of bone or muscle	Moderate hyperplasia	7	All subpharyngeal structures in bone and muscle
Trough 10	8	Entire aortic area including bone and muscle	Marked hyperplasia	6	Entire aortic area; invasion of bone and muscle	Hyperplasia	7	All subpharyngeal structures in bone and muscle
Trough 15	8	Entire aortic area including bone and muscle	Marked hyperplasia	6	Entire aortic area; invasion of bone and muscle	Marked hyperplasia	7	Entire subpharyngeal structures in bone and muscle
Trough 20	8	Entire aortic area including bone and muscle	Marked hyperplasia	6	Entire aortic area; invasion of bone and muscle	Marked hyperplasia	7	Entire aortic area; Moderate hyperplasia
Normal 20 above all ponds	Scattered follicles about aorta, filled with colloid	Normal		18	Scattered large follicles in aortic area, filled with colloid	Normal	19	Scattered large follicles in aortic area filled with colloid
Pond 1	8	Entire thyroid area packed with follicles, in bone and muscle	Marked hyperplasia	6	Entire aortic area filled; invasion of bone and muscle	Marked hyperplasia	7	Entire aortic area filled; invasion of bone
Pond 2	8	Entire thyroid area packed with follicles, in bone and muscle	Marked hyperplasia	6	Thyroid area filled	Moderate hyperplasia	7	Entire aortic area filled; invasion of bone
Pond 3	8	Entire thyroid area filled	Moderate hyperplasia	6	Thyroid area filled; invasion of bone	Moderate hyperplasia	7	Entire aortic area filled; invasion of bone
Pond 4	8	Entire thyroid area filled	Moderate hyperplasia	6	Thyroid area filled; invasion of bone	Moderate hyperplasia	7	Entire aortic area filled; invasion of bone
Pond 5a	8	Entire thyroid area filled	Moderate hyperplasia	6	Thyroid area filled; invasion of bone	Moderate hyperplasia	7	Entire aortic area filled; invasion of bone

TABLE II.—*Continued.*

Pond or trough No.	1909.		1910.		1913.	
	Condition of thyroid.		Condition of thyroid.		Condition of overgrowth.	
	Extent of overgrowth.	Classification.	Extent of overgrowth.	Classification.	Extent of overgrowth.	Classification.
Pond 5b	8 Entire thyroid area filled	Moderate hyperplasia	6 Thyroid area filled; invasion of bone	Moderate hyperplasia	7 Entire aortic area filled; invasion of bone	Moderate hyperplasia.
Pond 6	8 Entire thyroid area filled	Moderate hyperplasia	18 Entire thyroid area filled, in bone and muscle; infoldings; no colloid	Marked hyperplasia	19 Numerous small and large colloid-filled follicles in entire thyroid area	Colloid goitre.
Pond 7	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire thyroid area filled, in bone	Moderate hyperplasia	7 Aortic area filled; extension to bone	Moderate hyperplasia.
Pond 8	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire aortic area filled, in bone and muscle	Marked hyperplasia	7 Aortic area filled; extension to sub-mucosa	Marked hyperplasia.
Pond 9	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire aortic area filled, in bone and muscle	Marked hyperplasia	7 Aortic area filled; extension to sub-mucosa	Marked hyperplasia.
Pond 10	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire aortic area filled, in bone and muscle	Marked hyperplasia	7 Aortic area filled; extension to sub-mucosa	Marked hyperplasia.
Pond 11	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire aortic area filled, in bone and muscle	Marked hyperplasia	7 Aortic area filled; extension to sub-mucosa	Marked hyperplasia.
Pond 12	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire aortic area filled, in bone and muscle	Marked hyperplasia	7 Aortic area filled; extension to sub-mucosa	Marked hyperplasia.
Pond 13	8 Entire thyroid area filled, in bone and muscle	Marked hyperplasia	6 Entire aortic area filled, in bone and muscle	Marked hyperplasia	7 Aortic area filled; extension to sub-mucosa	Marked hyperplasia.
Pond 14	20 Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	18 Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	19 Numerous colloid-filled follicles in entire aortic area	Colloid goitre.
Pond 19	20 Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	18 Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	19 Numerous colloid-filled follicles in entire aortic area	Colloid goitre.

Pond or trough No.	1909.		1910.		1913.	
	Extent of overgrowth.	Condition of thyroid.	Extent of overgrowth.	Condition of thyroid.	Extent of overgrowth.	Condition of thyroid.
Pond 20	Entire subpharyngeal space filled, in bone and muscle	Colloid, moderate hyperplasia (hyperplasia involving)	18	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	hyper-19
Pond 21	Entire subpharyngeal space filled, in bone and muscle	Colloid, moderate hyperplasia (hyperplasia involving)	18	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	hyper-19
Pond 25	Entire subpharyngeal space filled, in bone and muscle	Colloid, moderate hyperplasia (hyperplasia involving)	18	Entire subpharyngeal space filled, in bone and muscle; visible goitre	Marked hyperplasia	hyper-19
Pond 26	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia, small visible goitre	30	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	hyper-31
Pond 32	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	30	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	hyper-31
Pond 35	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia, slight general fibrosis	30	Entire subpharyngeal space filled, in bone and muscle; visible goitre	Marked hyperplasia	hyper-31
Pond 36	Entire subpharyngeal space filled, in bone and muscle	Marked hyperplasia	30	Entire thyroid area filled, extension to skin, bone and muscle	Marked hyperplasia	hyper-43

1909.

1910.

1913.

1909.

1910.

1913.

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of food is the cause of the arrest of thyroid growth is the preservation of 250 forty-three months old fish which had been fed liver for the first twenty-one months of life and sea fish for the last twenty-two months. In these I found three out of the sixteen examined having distinct yellowish discoloration of the pharyngeal floor, and in the two specimens examined histologically the thyroid was completely involuted to the colloid state; but the amount and distribution were far more extensive than in the thirty-one and nineteen months old fish, showing that the thyroid overgrowth had progressed much farther before the involution began, and corresponds to the extra year of liver as food.

It is well known that foods influence the mammalian thyroid to a marked degree. Baumann (2) noticed that flesh diets stimulate the thyroid in dogs to active hyperplasia. I have repeatedly made such observations, liver being the most important food in this regard. Reid Hunt (3) in his acetonitrile experiments noted that liver stimulates the thyroid of white mice. Watson (4) has described the effect of different diets on rat thyroids and also noted that meats produce hyperplasia, while mixed diets do not. I have made similar observations. Dogs spontaneously recover from goitre on a mixed diet, while meats, especially liver, maintain hyperplasias. In zoological gardens where carnivores are held and bred in captivity and the diet is for the most part beef, goitre, rickets, and osteomalacic states are quite common. Foods modify the thyroid slowly, involution extending over months, while iodin involves hyperplasias much more rapidly. Iodin administered to dogs, sheep, pigs, and human beings often induces involution in a month, and in Lake Erie pike the involution of mild degrees of hyperplasia takes place in twenty-six days (5). In brook trout with extensive thyroid overgrowth and visible goitres involution occurs in forty days.

To summarize, it is seen that the ages of the fish held at the hatchery in August, 1913, are seven months, nineteen months, thirty-one months, and forty-three months. The seven months old fry all have active hyperplasia not different in degree or extent from the fry of 1909 and 1910. The nineteen, thirty-one, and forty-three months old fish all have completely involuted or colloid thyroids, and the amount and distribution of the thyroid tissue in the nineteen and thirty-one months old fish are the same and represent the degree of thyroid overgrowth reached during the first nine months of life when liver and heart only are fed. The forty-three months old fish had been fed with liver and heart for the first twenty-one months of life, and the thyroids both on gross and microscopical examinations were much larger and more widely distributed than in the nineteen and twenty-one months old fish.

### DISCUSSION.

These data afford supplementary proof for several of the conclusions deduced from the earlier work, and in addition establish another simple and certain means for the cure and prevention of goitre in fish.

The view previously held by some observers that this disease was closely related to true carcinoma is now only of theoretical interest,

since, in addition to the facts already reported, it establishes the fact that the feeding of fresh sea fish also readily arrests the disease. No tissue overgrowth that presents the biological phenomena described for this disease, which are also identical with those of mammalian goitre, can be considered malignant. It was urged by some observers that the reaction with iodin could not be utilized as proof that the disease was not cancer since arsenic, mercuric chloride, and colloidal copper were reported to have the same thyroid effects (6). I have not been able to affect these changes with arsenic in canine goitre. I (7) have also failed to modify the growth of the mammalian thyroid carcinoma by the use of iodin, and from an extensive experience with human thyroid adenomata the conclusion was reached that they also are rarely affected by iodin (8). On the other hand, simple physiological hyperplasias of all animals are readily and invariably modified by iodin.

This disease should be classified as endemic goitre or endemic thyroid hyperplasia. One must recognize all degrees of the overgrowth in fish, just as in mammals, from the slightest departure from normal, detectable only microscopically, to the most extensive infiltration of the thyroid region and the formation of external goitres. Whether this form of goitre is similar to ordinary goitre in birds and mammals is not known. As mentioned above, they are identical anatomically, physiologically, and pathologically in all their known reactions, but inasmuch as goitre is only the symptomatic manifestation of a nutritional disturbance it is possible that many agents are capable of exciting the thyroid to this single and only known anatomical manifestation of increased activity, compensatory hypertrophy and hyperplasia.

From our knowledge of mammalian goitre it would seem probable that the thyroid reaction in infectious diseases, puberty, pregnancy, cretinism, Basedow's syndrome, etc., are not due to the same exciting cause. The fundamental fault in nutrition is probably the same in all, but the immediate exciting agent is probably different. In mammalian goitre the localization in certain districts is most obvious, while in fish and in birds this is not so apparent although it is present. Goitre, however, occurs wherever favorable conditions are created whether in mammals or in the lower animals. It is

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therefore the increased susceptibility of mammals to goitre that brings out the localization of endemicity. Fish are very resistant to goitre and only acquire the disease when subjected to conditions incompatible with prolonged life in mammals.

The nearest approach to the etiology of goitre in fish was made when it was shown that food is the major etiological factor. Fish fed exclusively with liver always acquire thyroid hyperplasia, while fish of the same age, breed, and environment readily recover or escape goitre when fed with whole sea fish.

It is almost certainly a biochemical reaction, but whether it is due to the presence or absence of some substance normally needed by the organism is not known. A comparison of liver as a food with fish as a food seems to indicate that the liver lacked something normally needed by the developing fish which the diet of fish contained.

It is also probable that the liver contains some substances in excess, in attempting to utilize which, the animal exhausts other elements necessary for nutrition which are not present in the liver in sufficient amounts. As to the nature of this chemical fault one cannot reasonably speculate. From time to time most of the inorganic and many of the organic substances have been specified as etiological factors, but without foundation. Some observers have called it a toxin, but work based upon this hypothesis has added nothing to our knowledge of the etiology.

Iodin is certainly reduced in fish with thyroid hyperplasia, as in all other animals, and iodin certainly prevents and involutes hyperplasia, but the conclusion that goitre is due to a deficiency of iodin is not justified since there is considerable evidence to show that some other factor or factors are operating to divert or deplete an otherwise sufficient amount of iodin. On the other hand, the beneficial effects of feeding fish may be due to the iodothyreoglobulin that it contains, since only exceedingly small amounts are necessary, and the whole fish including the thyroid gland is used. Sea fish may also contain traces of iodin apart from the thyroid. Therefore much careful work must still be done to exclude iodin as the cause of the beneficial effects. I have made two iodin determinations on

mixed specimens of whole butter fish, and two determinations on mixed specimens of fish from which the thyroid areas had been removed. The results are as follows: (1) whole fish, slightest trace; (2) exclusive of thyroid area, no trace of iodin.

The possibility of a *contagium vivum* in fish goitre can, it seems to me, be eliminated since there is not the slightest evidence that it is either contagious or infectious. It obeys none of the laws which we associate with true infectious processes. No evidence of natural or acquired immunity has been obtained, though different species of fish exhibit different degrees of resistance (carp, trout).

Gaylord (9) has reported the finding of evidences of immunity, but this evidence does not exclude the following important factors: (1) Age is an important factor in all animals; the young are more susceptible to goitre and the old tend toward spontaneous recovery. (2) When the active hyperplasia involutes to the colloid state it is more difficult to produce again active hyperplasia (*a*) because of the increased age of the animal, and (*b*) because of the increased factor of safety due to the increased number of thyroid follicles over what obtains normally. For example, if a dog has fifty grams of colloid thyroid it is more difficult, apart from any age factor, to induce active hyperplasia in it than if the dog has three grams of normal thyroid; but other things being constant, three grams of colloid will undergo hyperplasia as readily as three grams of normal thyroid. (3) The food is probably the most important factor in determining the onset or cessation, progression or regression of the hyperplasia. (4) Water supply has some influence on the resistance of fish to active hyperplasia, reduced and much used water favoring, and large amounts lessening it. (5) The different Salmonidæ vary greatly in their susceptibility. I have seen none that were not susceptible, but the brown trout and rainbow trout in my experience have been more resistant than the silver salmon or brook trout.

The water supplied to this hatchery has little if anything to do with the development of goitre, since the fish have never developed the disease unless the single factor of overfeeding with the highly abnormal diet of liver and heart is also operating. The following observations bearing on the relation of water to the etiology and spread of goitre may be mentioned: (1) Within the hatch house are

twenty-one troughs,—twelve hatch troughs and nine nursing troughs. The twelve hatch troughs receive water directly from the spring, while the nine nursing troughs receive the water from the twelve hatch troughs. Every summer these troughs are used to hold extra fry. They are kept remarkably clean, yet all the fry have shown equally marked thyroid overgrowth in each of the years that they have been examined. Fish living outside the troughs and ponds (hence not directly fed with liver), whether above, below, or between the troughs and ponds, never develop active hyperplasia, although in most instances they have some degree of colloid goitre, showing that they had once been confined in the troughs or ponds and that, after escaping, the hyperplasia involutes. (2) The 1913 distribution of the fish made another observation possible. In house II (text-figure 1) there are 3,000 nineteen months old fish which have been fed with fish for the past ten months; all these fish have colloid glands. While above in house I and below in house III there are seven months old fry which have been fed only with liver and heart muscle and all have well marked active thyroid hyperplasia. There is also some evidence that liver when fed in excessive amounts is a more potent stimulus than when fed in small amounts, as the fish living in the runways between the houses also obtain small particles of liver which float through the screens but do not develop active hyperplasia. Plehn (9) has reported greater variations in the occurrence and distribution of the disease in a fish hatchery than they could account for purely on the basis of external changes. They suggest that these variations point to infection. I have also seen wide variations in its occurrence in different hatcheries and even in the same hatchery, but feel that all the variations could be accounted for on the basis of one or more of the following factors: (1) food, (2) water supply, (3) age, and (4) species of fish. Some observers have applied the term epidemic goitre to this form of goitre in fish. This is wrong. There is nothing acute or localized in its occurrence or distribution. It appears wherever fish are overfed with liver or other incomplete foods. It extends over the entire time of such feeding and all fish are affected.

Goitre has been present in this hatchery for the past twenty-five

years and, in my opinion, it will remain as long as fish of any age are fed with liver.

*Treatment and Prevention.*—Trout living in their normal environment and partaking of their normal food do not develop goitre, hence in the treatment and prevention our efforts should be directed toward imitating natural conditions as far as possible. However, as with many other animals it has been found possible to alter these natural environmental conditions within extremely wide limits without seriously impairing their health. Goitre is one of the serious diseases that has developed in testing the range of adaptability. The infectious diseases of fish, as of mammals, are better known and methods for the cure and prevention have been studied longer. Goitre belongs to the group of diseases dependent upon nutritional disturbances, and our knowledge of the food requirements of animals generally and of fish in particular is as yet in its infancy.

It has been conclusively shown that the feeding of the highly artificial and incomplete diet of liver and heart muscle is the major factor in the causation of fish goitre, and the first essential in treatment is to provide some other food that meets the animal's requirements. A natural food of trout is fish and the experiments of the past two years show that when sea fish is fed to these trout existing goitre is cured and the development of goitre is wholly prevented.

The feeding of fish with hard fine bones or with large heavy scales is associated with risks mentioned above. The fish stomach is adapted for the ingestion of relatively large food boli, and food should be fed in as large masses as they will take since digestion is a continuous process in fish. This would probably solve the bone question and would tend to control overfeeding. Some sort of chopper should be devised instead of hashers, since hashers mince the food and allow of too rapid digestion and, therefore, too long intervals of stomach inactivity. Coarse fish, like sharks, rays, etc., might be utilized. Sea fish, therefore, makes a perfect food as far as the cure and prevention of goitre are concerned. Overfeeding must be guarded against. Definite amounts of food should be fed but once a day with a fast day each week comparable to the fast day maintained among the carnivores at Zoölogical Gardens. No obser-

vations have been made as to the minimum amounts of sea fish necessary to involute or prevent hyperplasia, but judging from the rapidity of the involution when the food is changed to fish only, it would appear that the daily feeding with fish was not necessary to cure or prevent the hyperplasia. It might be beneficial to alternate between fish and liver or between fish and other foods daily or weekly. Overcrowding is dependent on the water supply and the food. It is a highly important factor as regards the general health of the fish, the prevention of traumata and infectious diseases, the oxygen supply, etc., but is only a minor factor in the production of goitre.

Cleanliness likewise is a secondary factor in goitre, but is of great importance in the prevention of other diseases and as a general hygienic measure cannot be neglected. Modification of pond construction with this in view would greatly simplify the cleaning and sanitary problems. Iodin in minute traces has also been found to prevent and cure goitre. Indeed it is a specific therapy for goitre but does not relieve other untoward manifestations of an incomplete food. Gaylord (6) has reported that arsenic and mercuric chloride also effect similar changes in fish thyroid hyperplasia. Our observations with arsenic in canine goitre were negative and in any event the action of arsenic and mercuric chloride is not comparable to the action of iodin on the thyroid.

#### CONCLUSIONS.

1. Goitre in fish is a non-infectious, non-contagious, symptomatic manifestation of a fault of nutrition, the exact biochemical nature of which has not been determined.
2. Feeding the highly artificial and incomplete diet of liver is the major etiological factor in bringing about this fault of nutrition which is at once corrected by feeding whole sea fish.
3. Water plays no essential part in the etiology, transmission, or distribution of the disease in the fish of this hatchery.

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## EXPLANATION OF PLATES.

## PLATE 13.

FIG. 1. Rough projection sketch of a transverse section through the thyroid area of a brook trout with a normal thyroid.

FIG. 2. Rough projection sketch of a transverse section through the thyroid area of a brook trout with a mild degree of active hyperplasia.

FIG. 3. Rough projection sketch of a transverse section through the thyroid area of a brook trout with a mild degree of colloid (goitre) gland.

## PLATE 14.

FIG. 4. Transverse section of the thyroid area of a five months old brook trout showing the extent of the thyroid overgrowth at this age in fry of the hatch of 1910.

FIG. 5. Higher magnification of figure 4, showing the filling up of the thyroid area, the extension to the cuticle, to the pharyngeal mucosa, and on the right the extension to the gill filaments. Note the relation of the follicles to the two terminal branches of the ventral aorta, the size of the follicles, the high columnar epithelium with infoldings and plications, and the absence of stainable colloid.

## PLATE 15.

FIG. 6. Transverse section of the thyroid area of a seven months old brook trout of the hatch of 1913 showing the pharyngeal mucosa above and gill filaments laterally. Note the distribution of the hyperplastic follicles about the ventral aorta and their irregular distribution throughout the thyroid area.

FIG. 7. Transverse section of the thyroid area of a seventeen months old brook trout, showing the whole thyroid area uniformly filled with hyperplastic thyroid follicles.

## PLATE 16.

FIG. 8. Higher magnification of an area from figure 7, showing the infoldings and plications, vascularity, high columnar epithelium, absence of stainable colloid, and the great distortions and variations in the size of rapidly growing thyroid follicles.

FIG. 9. Transverse section of the thyroid area of a nineteen months old brook trout which during the first nine months of life had active hyperplasia, and upon the introduction of fish as the food all the follicles involuted back to the colloid state. Note the distribution and numbers of follicles as those attained during the stage of active hyperplasia, while the individual follicles now resemble in all essentials normal follicles. Note also the extension into bone and muscle. This is the colloid, or resting, or cured stage of a mild degree of active hyperplasia.

## PLATE 17.

FIG. 10. Higher magnification of a portion of figure 9, showing part of the ventral aorta and the large venous space to the left. Compare the numbers and distribution of the follicles, their colloid contents, and their epithelial investments with those of figure 12.

FIG. 11. Transverse section through the thyroid area of a nineteen months old brook trout, showing the pharyngeal mucosa above, gill filaments laterally, and scattered thyroid follicles of different sizes widely distributed about the ventral aorta. Normal thyroid.

FIG. 12. Higher magnification of an area from figure 11, showing the size and arrangements of the follicles together with the colloid contents and epithelial investments.

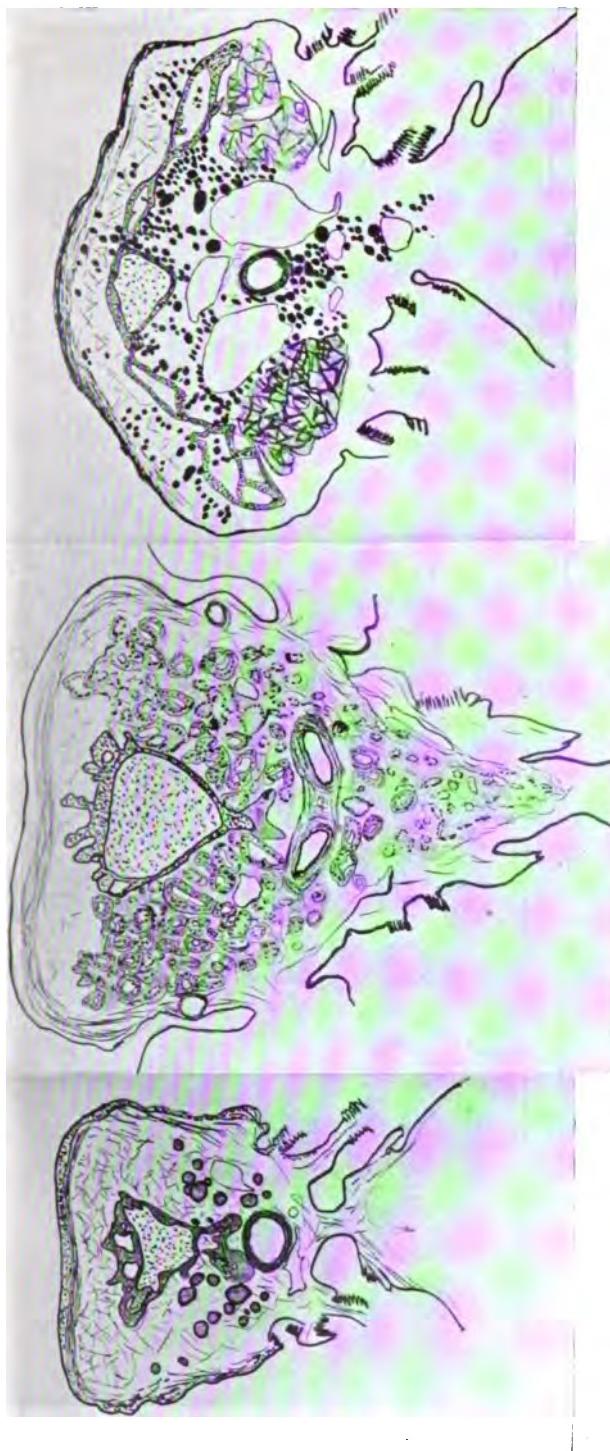


FIG. 3.  
(Marine: Goitre in Brook Trout.)

FIG. 2.  
FIG. 1.

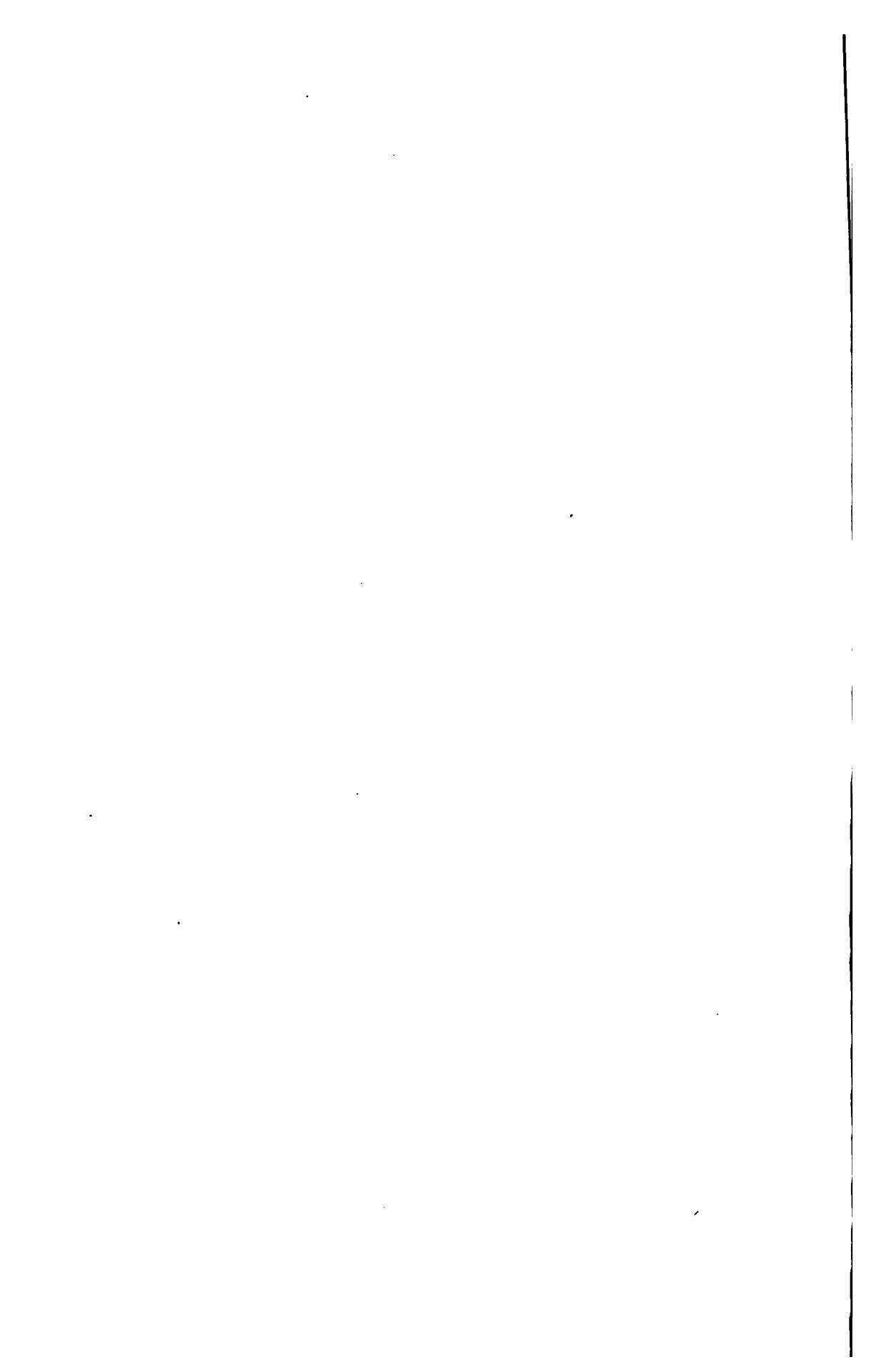




FIG. 4.

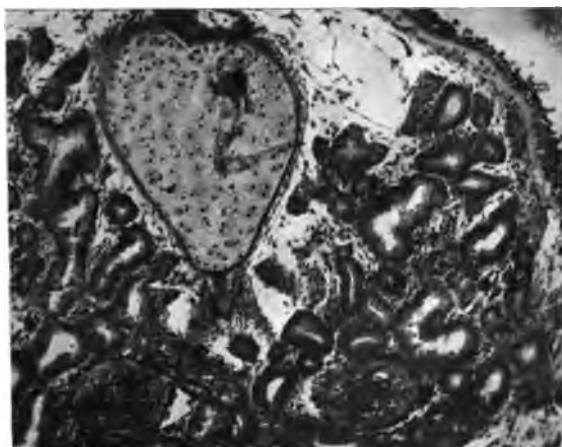


FIG. 5.

(Marine: Goitre in Brook Trout.)



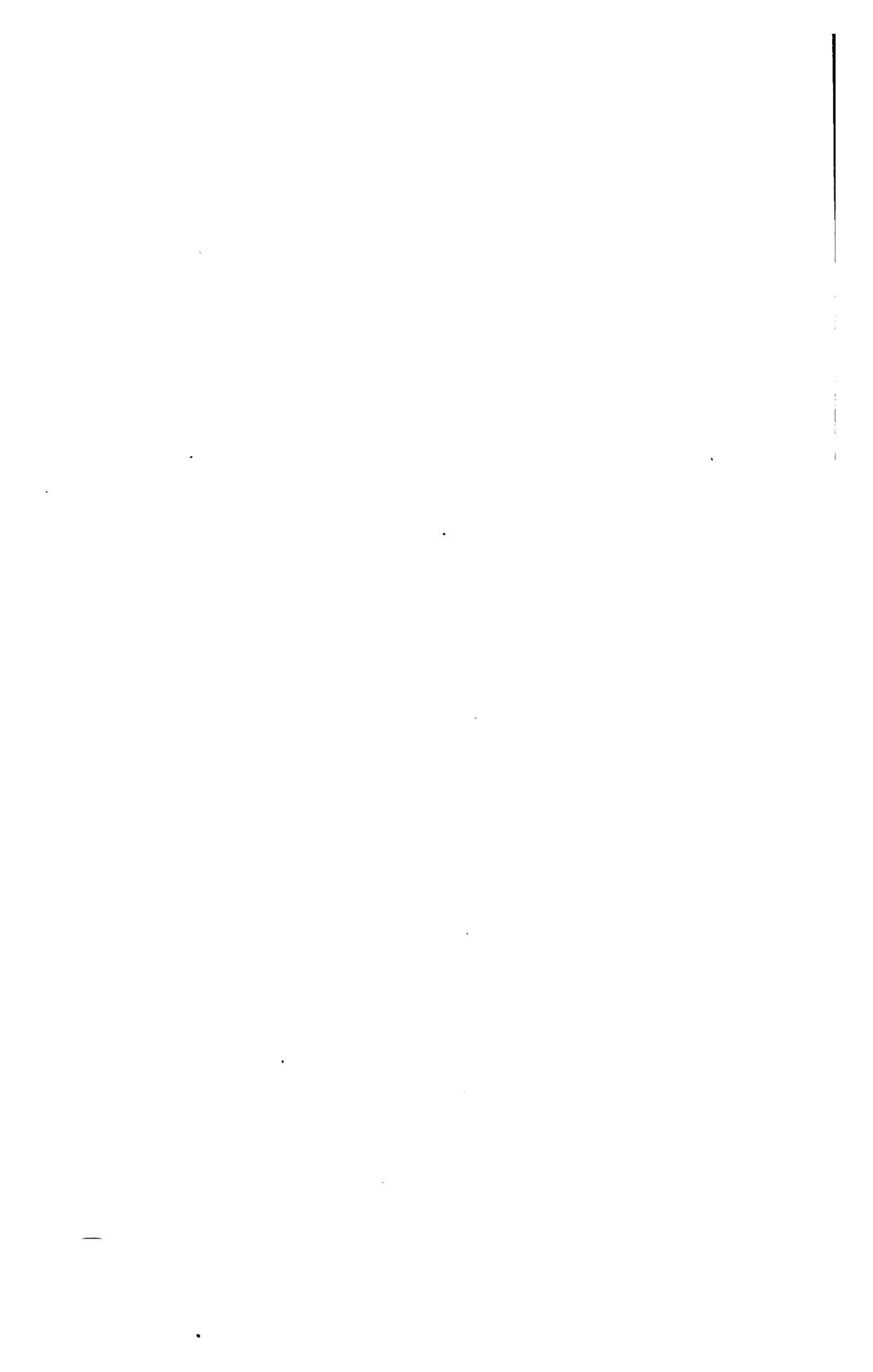


FIG. 6. j



FIG. 7.

(Marine: Goitre in Brook Trout.)



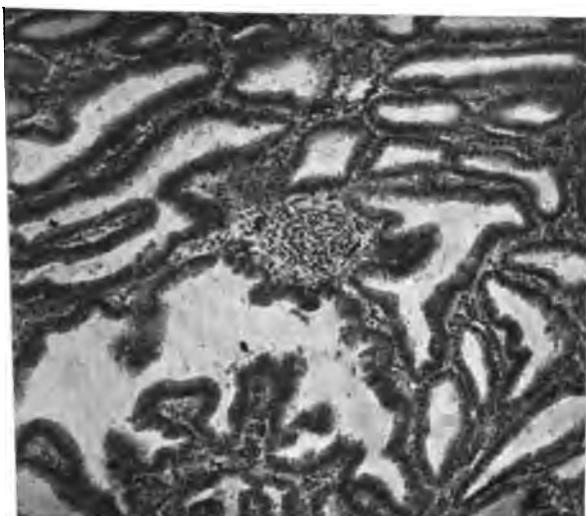
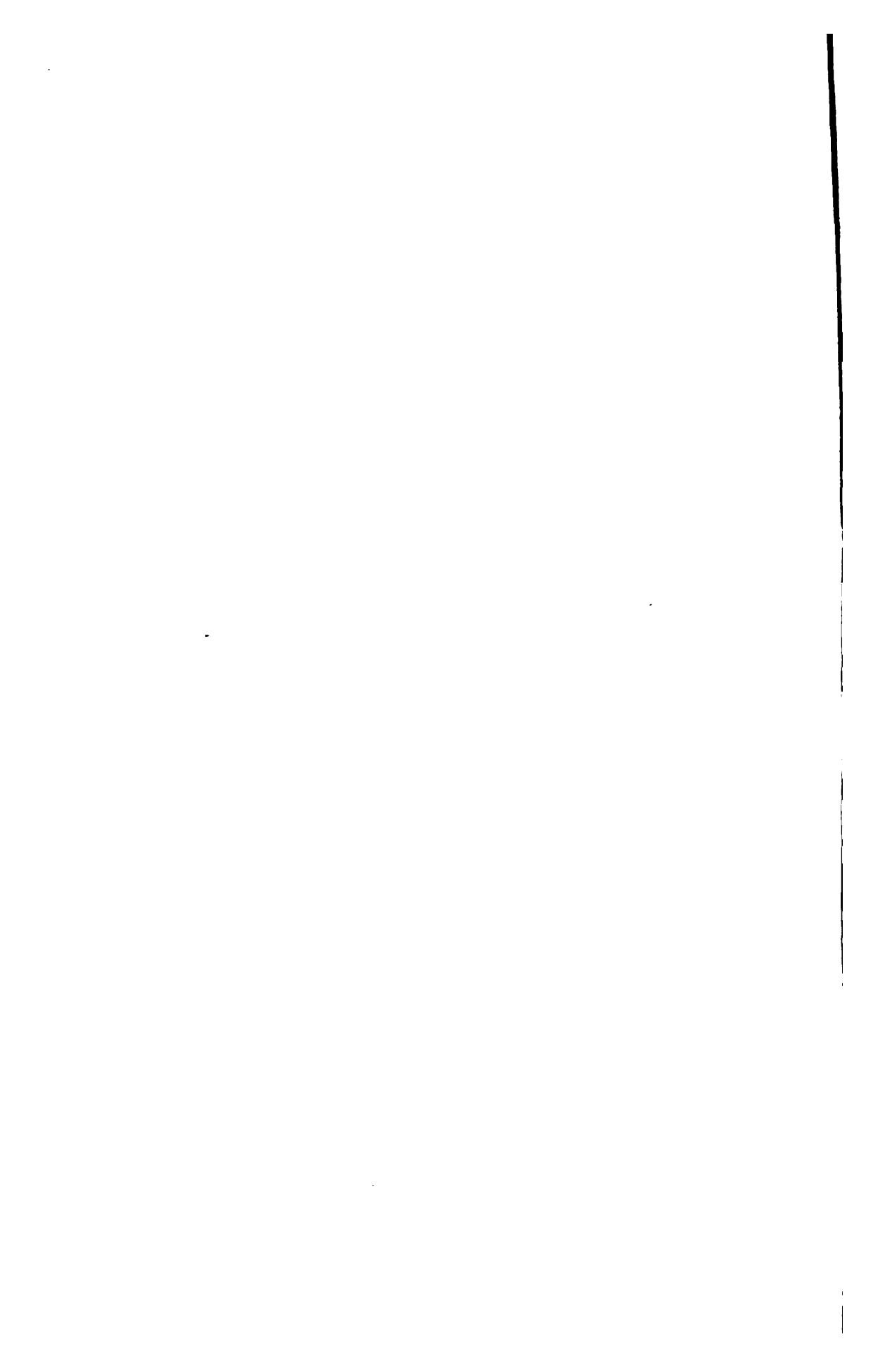


FIG. 8.



FIG. 9.

(Marine: Goitre in Brook Trout.



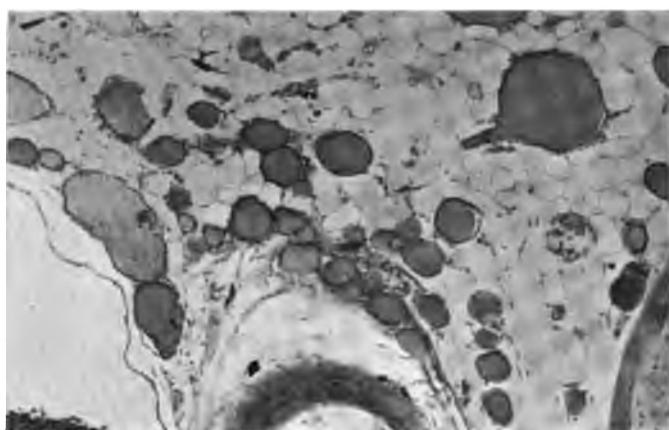


FIG. 10.



FIG. 11.

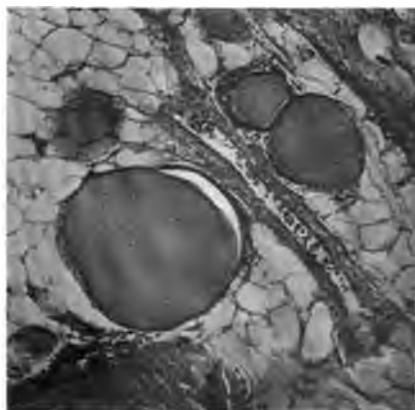
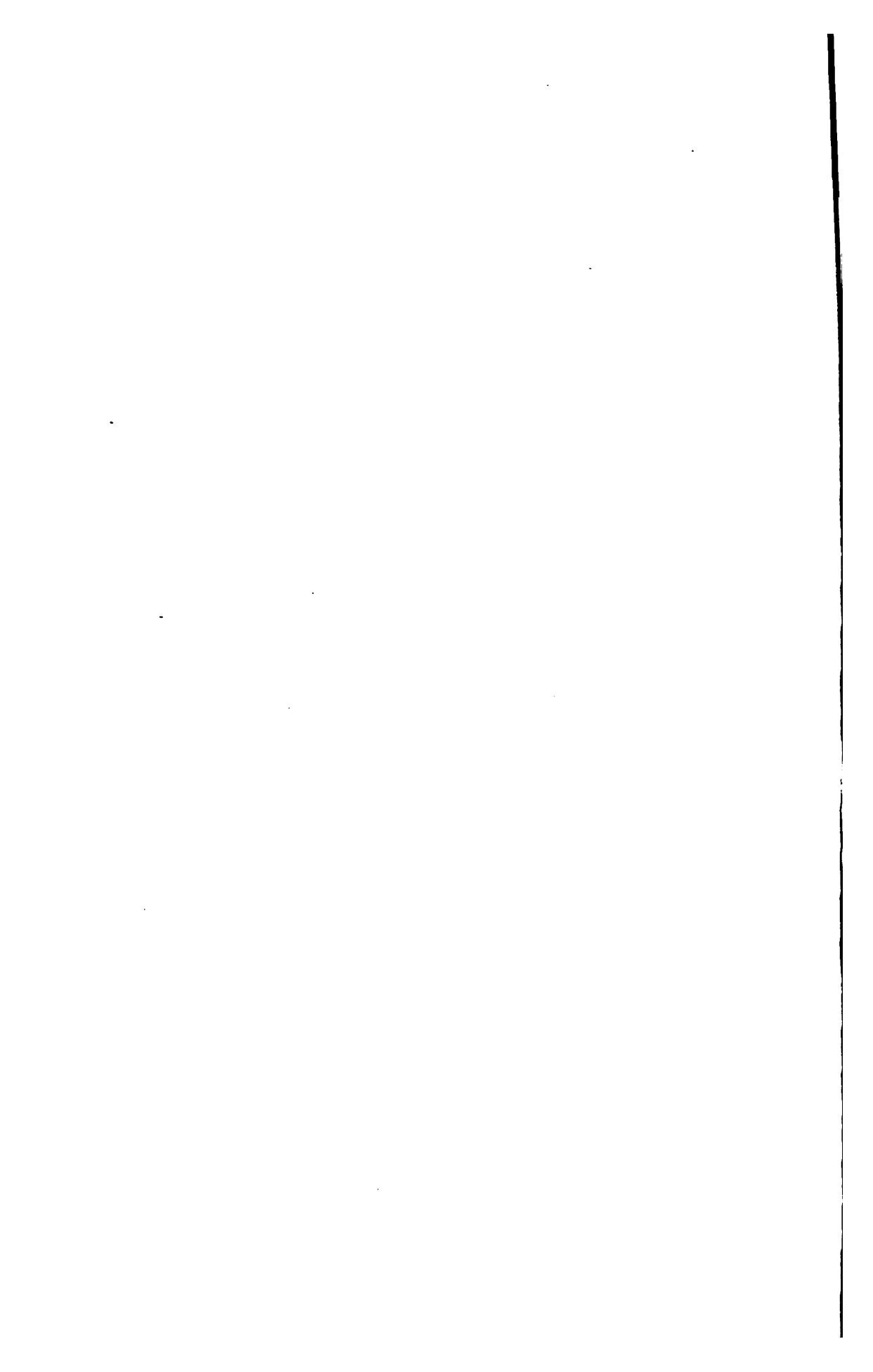


FIG. 12.

(Marine: Goitre in Brook Trout.)



## OBSERVATIONS ON TETANY IN DOGS.

RELATION OF THE PARATHYROIDS TO THE THYROID; RELATION OF  
TETANY TO AGE, AMOUNT OF PARATHYROID TISSUE REMOVED,  
ACCESSORY PARATHYROIDS, PREGNANCY, LACTATION,  
RICKETS, SULPHUR, AND DIET; RELATION OF  
PARATHYROIDS TO SUGAR TOLERANCE;  
EFFECT OF CALCIUM SALTS.\*

By DAVID MARINE, M.D.

(From the H. K. Cushing Laboratory of Experimental Medicine of Western Reserve University, Cleveland.)

### THE DISTRIBUTION OF PARATHYROID TISSUE AND THE RELATION OF THE PARATHYROIDS TO THE THYROID.

As a complicating factor in thyroid work during the past eight years I have observed thirty-eight cases of tetany and three instances where total removal of the thyroid lobes was not followed by any of the phenomena of tetany.

The frequency of tetany following thyroidectomy in dogs is an index to the constancy of location of the parathyroid glands. On account of the fact that in most of these cases partial thyroparathyroidectomy was done from time to time extending over months and sometimes years before the insufficiency became clinically detectable, ample opportunity was offered for compensatory adjustment, and in this way some cases escaped that would otherwise have had at least a transient tetany if the same amount of parathyroid tissue had been removed suddenly.

In all carnivora it is exceedingly difficult to remove totally even the normal thyroid without obtaining at least a transient tetany on account of the intimate anatomical relation of the parathyroids to the thyroid, while in man and the herbivora this difficulty rarely arises on account of the wide separation of the inferior or external or IIId parathyroids from the thyroid lobes. In dogs the difficulty is of course greatly increased when the thyroid lobes are enlarged, as they are in about 90 per cent. in this locality. There is much writ-

\* Received for publication, October 9, 1913.

ten concerning the distribution of the parathyroid tissue. Its origin from four separate anlagen enhances the possibilities for abnormal distribution outside the thyroid area. In dogs aberrant parathyroids are most frequently found in connection with the thymus. In one series of routine examinations of ninety-eight thymuses, I accidentally included parathyroids in the specimens for histological examinations on two occasions and in a case of transient tetany following removal of both lobes of the thyroid I found at post-mortem examination 257 days later a large accessory parathyroid in the thymus. In the three instances (A-106, A-123, and A-194) where following complete removal of both lobes not even transient tetany developed, no parathyroid tissue was found at the routine post-mortem examination, though several suspected bits of tissue were examined histologically.

Parathyroid tissue unconnected with the thyroid lobes in sufficient amounts to maintain life after the removal of both thyroid lobes is present in at least 5 to 6 per cent. of dogs. This percentage is further increased (1) by partial parathyroidectomy extending over months and years which allows of the development of any compensatory mechanism, and (2) by the temporary use of calcium salts which will occasionally tide the animal over an otherwise fatal tetany.

These facts concerning the distribution of parathyroid tissue, and the influence of compensatory hypertrophy and calcium salts are of importance in the controversy that has arisen as to the relation of the parathyroids to the thyroid and their importance to life.

Bearing on the question of the interrelationship of the parathyroids and thyroid which some English observers, notably Vincent (1) and Forsyth (2), have supported are the only two cases of operative myxedema in dogs we have observed (both transient), and in both of these transient and partial tetany not associated with the myxedema developed. The cases are given below.

*Dog A-112.*—Female fox terrier. Jan. 11, 1908. Weight 5.5 kilos. Jan. 15. Entire left lobe and two thirds of right lobe (weight 4.15 gm.) removed. Mar. 15. Two pups born. Mar. 25. Removed about one half of remaining portion of right lobe. Mar. 26. Violent tetany. Calcium chloride and milk given daily until Apr. 6. Oct. 29. Five pups born. Nov. 3. Violent tetany. Milk and

calcium chloride given for three days; tetany cleared up. Dec. 3. Pups weaned. Dec. 7-18. The remainder of the right lobe (weight 0.25 gm.) was removed, and 10 c.c. of saturated aqueous solution of calcium chloride were given daily. Dec. 24. Hair falling out, skin dry and itchy. Weight 5.9 kilos. At first it was thought to be mange and the dog was given sulphur rubs, but during January, 1909, alopecia became very marked and the dry skin was everywhere visible. Jan. 29. Weight 7.2 kilos. Feb. 12. Weight 7 kilos. New coat of hair is beginning to come in. Feb. 26. Weight 6.2 kilos. Mar. 15. Weight 6.1 kilos. New coat of hair about 0.5 cm. long. In heat. May 24. Two pups born. June 5. Weight 5.7 kilos. July 10. Weight 6 kilos. Aug. 10. Weight 6.2 kilos. Sept. 14. Weight 6.2 kilos. Oct. 4. Weight 5.9 kilos. Nov. 26. Weight 5.9 kilos. Dec. 9. Killed in fight with another dog.

*Autopsy.*—One large accessory parathyroid was found in the thymus, and three small aortic thyroids were in a state of marked hyperplasia.

*Dog A-183.*—Female mongrel. Nov. 16, 1909. Weight 10 kilos. Nov. 16-23. 1 c.c. of Lugol's solution given. Dec. 1. Bit of left lobe removed. Weight 0.45 gm. Dec. 1, 1909, to Feb. 9, 1910. Dog received 1 gm. of sodium cyanate daily. Dec. 15. Another bit of left lobe was removed. Weight 1.25 gm. Jan. 6, 1910. Remainder of left lobe removed (weight 2.49 gm.). Jan. 19. Bit of right lobe removed (weight 0.67 gm.). Feb. 9. Bit of right lobe removed (weight 1.58 gm.). June 13. Weight 10.2 kilos. Oct. 11. Nine pups born. Nov. 15. Pups weaned. All have strikingly enlarged thyroids. Oct. 15. Weight 12 kilos. Hair falling out. Jan. 6, 1911. Loss of hair is marked. Weight 12.2 kilos. Feb. 12. Weight 11 kilos. New hair coming in. Mar. 3. Weight 10.2 kilos. Apr. 1. Weight 9 kilos. Active; coat glossy. Apr. 11. Remaining portion of right lobe isolated, about size of a medium olive, much hypertrophied. Removed portion (weight 4.6 gm.) leaving a small stump. Apr. 13. Violent tetany. Calcium chloride solution by stomach. Apr. 18. Weight 9.6 gm. Occasional tetany sometimes mild and often severe, always controlled by calcium lactate. May 1. Impregnated. May 20. Pregnant. Suddenly taken with violent tetany; relieved by calcium lactate. Occasional twitchings until June 30, nearing full term, severe tetany, calcium lactate. July 1. Tetany again, calcium lactate. Was in tetany daily until July 6, when she died of tetany shortly after the birth of seven living full term pups.

Histological examination of the remaining stump of the thyroid lobe revealed a trace of parathyroid tissue in the dense scar tissue of the thyroid capsule. The thyroid tissue is in a state of marked hyperplasia. Two aortic thyroids are present, but no other parathyroid tissue is found.

In these two experiments the tetany was dissociated from the myxedema. In dog A-112 the last attack of tetany preceded by about forty days the onset of myxedema which occurred following the last operation for total removal of the thyroid lobes. The myxedema cleared up in three months and the dog remained in apparent health clinically until killed on December 9, thirteen months after the last attack of tetany and nine months after the disappearance of myxedema. In dog A-183 the myxedema developed after the fifth

operation of partial removal of thyroid (about nine tenths), and completely cleared up two months before the onset of tetany on the day after the sixth operation for removal of 4.6 grams of the much hypertrophied stump of the right lobe. The dog remained subtetanic with the aid of calcium salts until the end of pregnancy, when an uncontrollable parathyroid insufficiency developed and ended fatally.

Such observations when compared with the experiences of others,—Simpson (3) with tetanies in sheep; Halsted (4), MacCallum (5), Erdheim (6), and others with tetanies in dogs; Kocher (7), Reverdin (8), and Fuhr (9) with total thyroidectomy in man; Maresch (10), Erdheim (11), and Getzowa (12) with congenital defects; and Roussy and Clunet (13) with spontaneous myxedema,—leave no doubt that as regards myxedema and tetany the parathyroids and thyroids are anatomically, physiologically, and pathologically independent structures.

**THE RAPIDITY OF THE DEVELOPMENT OF TETANY AND THE RELATION  
OF TETANY TO AGE, AMOUNT OF PARATHYROID TISSUE REMOVED,  
ACCESSORY PARATHYROIDS, PREGNANCY, LACTATION,  
RICKETS, SULPHUR, AND DIET.**

I have seen violent tetany develop as early as seven hours after the removal of the parathyroids (dogs A-149 and A-167) and end fatally within eleven hours of the operation. In our series the average time between the last operation and the onset of tetany was eighteen to twenty-four hours. This has impressed me as relatively more rapid than the average time intervals reported by other observers. The conditions are not quite comparable as usually the parathyroidectomy is made at a single operation, while in nearly all of our cases partial thyroparathyroidectomy had been practiced for weeks and months and occasionally a year before the final operation precipitating the tetany. It is therefore possible that the factor of safety or protective reserve was greatly reduced before the final operation.

Several factors influence the rapidity of onset of tetany: (1) age, (2) the amount of parathyroid tissue removed, (3) the presence of inaccessible accessory parathyroid tissue, (4) pregnancy and lactation, (5) rickets, (6) the use of sulphur, and (7) the diet.

*Age.*—The age of the animal, as many observers have pointed out, is important. Simpson (3) in his work with sheep showed that lambs usually develop tetany following parathyroidectomy, while in yearlings he never observed a fatal tetany, and in old sheep there were no tetanic manifestations at all. In dogs and cats tetany follows the parathyroid removal at any age so that the age factor cannot be shown in this way. The young are, however, more susceptible. The difference due to age is suggested by the following observations. I ligated the superior thyroid arteries on both sides in four pups (A-203, A-204, A-205, and A-206) of the same age (sixty-two days) and same litter, and in all violent tetany developed on the following day, eighteen hours after the ligations. They died of tetany on the eighth, twelfth, ninth, and eighth days, respectively, although calcium lactate was given daily. At autopsy the wounds were healed *per primam*. Only the regions of the superior poles had been traumatized by the operative procedure. The thyroid lobes were somewhat enlarged in each case and in a state of marked hyperplasia. Anemic necrosis roughly involving the upper half of the lobes was present in each case. The inferior thyroid artery, which is normally very small in the dog, was intact and uninjured in all. In twelve similar operations in adult dogs undertaken to study the effect of superior thyroid artery ligation none developed tetany although there was in all a small zone of necrosis at the upper poles involving in some instances a third of the lobe. While the difference in outcome between young pups and adults may be explained principally on the difference in the extent of the necrosis still this can scarcely be the whole explanation. These pups were slightly rachitic and this also is probably an important factor.

*Amount of Parathyroid Tissue Removed.*—In adult dogs under normal conditions of food and environment one uninjured IIId parathyroid is sufficient to prevent fatal tetany. Many cases are reported where a IVth, or even smaller bit of parathyroid tissue has prevented all signs of tetany. I have seen three cases (dogs A-106, A-123, and A-194) where total removal of the thyroid lobes with their normal quota of parathyroids induced no symptoms. The routine autopsies of these cases failed to reveal any accessory para-

thyroid tissue although several suspected bits were examined histologically. The examinations were in no sense complete, as such a procedure would involve serial sections of the entire neck and upper thorax, and I am convinced that accessory parathyroids were present somewhere in the parathyroid area. On the other hand, I have seen typical and violent tetany occur in a lactating bitch, where four parathyroids were intact, as ascertained at autopsy.

It is clear from these extremes and from the larger number of cases with intermediate amounts of parathyroid tissue showing variations in the occurrence of tetany that other factors than quantity of parathyroid tissue determine the onset of tetany. Thus when an anatomical insufficiency exists there exists also a physiological insufficiency, but the reverse is often not true since a physiological or relative insufficiency may exist when two and even four parathyroids are intact.

The same is, of course, true of the thyroid tissue,—that where an anatomical deficiency exists a physiological insufficiency exists also, but in the spontaneous thyroid insufficiencies and even in the induced insufficiencies there is usually an excess of thyroid tissue in the early stages.

*Pregnancy and Lactation.*—These factors tend to induce a relative parathyroid insufficiency. In 1888 Halsted (4) observed this in his cases of partial parathyroidectomy in dogs which developed tetany late in pregnancy. Since then many workers have observed this tendency. There were five cases in this series (dogs A-112, A-113, A-132, A-161, and A-183) where tetany developed with pregnancy or lactation.

In dog A-112 partial thyroparathyroidectomy, followed by a transient tetany for four days, was done six months before the next pregnancy. The dog developed tetany early in lactation which was controlled by calcium salts. The remainder of both lobes was removed later without causing tetany, calcium salts being given for eleven days. Six months later the animal gave birth to another litter of pups without any tetany resulting.

In dog A-183 a mild tetany continued throughout the pregnancy, which was controlled until the last week by the use of calcium salts.

Then in spite of the daily use of calcium lactate the tetany became severe and terminated fatally at the birth of the full term pups. Another bitch (A-113) aborted during a severe tetany that probably could have been prevented by calcium salts.

In another case (A-132) following a first operation for the removal of one and three fourths thyroid lobes on December 7, 1908, a transient tetany developed which was controlled by the administration of calcium salts for ten days. The dog remained free from tetany during the next pregnancy and lactation (May 1 to August 1, 1909) but developed tetany on April 1, 1910, thirteen days before the birth of a second litter of pups. This was wholly controlled by calcium lactate until the birth of the pups, then crushed bone was substituted which probably prevented tetany during lactation. She remained in apparently good health for forty days after weaning the pups and was used for other experiments.

In another case (A-161) violent tetany developed during lactation with at least four parathyroids intact, as was determined later by operation and autopsy. The regular diet of cooked meat with bread but no bone had been used. A synopsis of the protocol follows.

*Dog A-161.*—Female mongrel; pregnant. Mar. 17, 1909. Thyroid lobes palpable. Sulphur and lard to be given daily (50 gm. of lard containing 5 gm. of powdered sulphur). Weight 9 kilos. Mar. 18. Bit of left lobe, lower pole (weight 0.4 gm.) removed for histological examination. Mar. 21. Stitches removed, wound healed; protective bandage. Mar. 27. Five pups born. Average weight 195 gm. Apr. 2. Weight 7.5 kilos. Stools black and tarry; slight diarrhoea. Apr. 13. Weight 7.2 kilos. Dog is losing weight steadily. This morning a typical and severe tetany developed. At examination the dog is lying on the right side, legs extended, respirations 154. Pulse with stethoscope 224. Marked salivation. Pupils dilated, palpebral fissures also widened. Dilatation of the peripheral vessels. Ears, nose, paws, and skin surface generally hot to the touch. Temperature 104° F. All muscles spastic with fine constant tremors and rapidly recurring coarse jerky twitchings of muscle groups all over the body, in the legs, back, jaws, neck, etc. No obvious gastric dilation. Received one dose of 40 c.c. saturated aqueous solution of calcium chloride by stomach tube, and in twenty minutes she was free from general symptoms of tetany, and able to walk but weak. Sulphur stopped. During the afternoon of the same day another bit of the left lobe, lower pole (weight 1.33 gm.) was removed. The thyroid had enlarged considerably since the last operation. A small IVth parathyroid was attached to the bit of thyroid removed. The dog rallied quickly and ate heartily in the evening. Apr. 14. In excellent condition. Apr. 17. In excellent condition.

Nursing four pups, the other one having been killed at birth as a control. Average weight of the pups is now 755 gm. Apr. 21. Bandage and stitches removed, wound healed, protective bandage. Weight 6.8 kilos. Apr. 22. Started sulphur again, 5 gm. daily. Apr. 29. Definite though mild tetany. Dull but able to stand up, gait ataxic, jaws, legs, back, and head constantly twitching. No tachypnea. Pulse 130. Sulphur stopped but no calcium given or any change in daily routine. Apr. 30. Free from tetany. May 6. Pups weaned. The dog has remained free from any outward signs of tetany, and in the afternoon in attempting operation she choked up with mucus and died in spite of attempts at resuscitation.

*Autopsy.*—Performed at once. Trachea blocked with mucus. Thyroid lobes enlarged. The remaining part of the left lobe weighs 5.22 gm. The intact right lobe weighs 7.92 gm. The two large external or IIId parathyroids are normally placed. The IVth parathyroid on the right lobe is also prominent. No other parathyroid tissue is found. Thymus large and persistent. Spleen normal. Adrenals, liver, kidneys, and lungs, appear normal. No accessory thyroids could be seen or felt in the aortic area.

*Histological Examination.*—The specimen of thyroid removed on Mar. 18 showed colloid early hyperplasia; the specimen removed on Apr. 13 showed colloid early hyperplasia; and the specimen removed on May 6 showed colloid moderate hyperplasia.

The parathyroid cells are large and more distinct than normally. There is no evidence of hyperplasia.

*Summary.*—The bitch developed violent tetany twenty-four days after starting sulphur and seventeen days after the birth of five healthy pups. A single administration of calcium chloride relieved the tetany at once. The sulphur was stopped for nine days following the tetany and then started again. Seven days later the dog developed mild tetany, and on stopping the sulphur no further evidence of tetany developed during the next seven days.

It may be stated, therefore, that in dogs as in man tetanies tend to develop late in pregnancy and occur even more frequently during lactation. The most important question is, Are these pregnancy and lactation tetanies identical fundamentally with those due to parathyroid extirpation? Several authors have answered this question affirmatively. In the cases that I have observed in dogs there are no objective differences to be made out. They present the same clinical manifestations, though usually milder, and they react with calcium salts in the same way.

Since the discovery by MacCallum (14) and Parhon and Ureché (15) of the effect of calcium on parathyroid tetany, it has been suggested that pregnancy and lactation predispose to tetany because at these times the mother suffers an increased depletion of mineral salts among which is calcium. This view draws support from the facts that calcium salts permanently relieve such tetanies, that feeding

foods rich in lime salts or giving lime salts during a second pregnancy will prevent tetany in animals that during their earlier pregnancies developed tetany, and that tetany develops very quickly (six to twelve hours) after removing the parathyroids in lactating or recently lactating animals (Carlson (16)).

I am inclined to think that the administration of large amounts of sulphur favors the development of tetany. From a considerable experience with sulphur in relation to thyroid hyperplasia I came to the conclusion that those dogs which had been given sulphur (and most of our tetany cases had had sulphur) for three to four weeks developed tetany earlier, more violently, and possibly in the presence of more intact parathyroid tissue than otherwise. The case cited above (dog A-161), which cleared up so quickly when sulphur was stopped, reappeared after it was started again, and again cleared up after it was stopped, other conditions remaining constant, was further evidence that the administration of sulphur over long periods of time aids to some extent in bringing about a state of nutrition favoring the development of tetany. How it operates is of course mere speculation. Sulphur is not wholly inert. The formation of sulphides in the intestine and the slight laxative effect suggest that possibly some of the alkaline earths are withdrawn from the field of absorption by this element.

*Influence of Foods on Tetany.*—Meats aggravate the symptoms, and milk, bone, and other foods rich in calcium are protective against tetany or delay its onset. I have seen several instances where bone administration delayed the onset of tetany. No other substance besides calcium has been found to have this effect. Similar effects of an extremely mild and probably more complex nature have been claimed for magnesium and strontium, but in our hands they have yielded negative results. It is certain that tetany is a vastly more complex nutritional disturbance than a calcium deficiency. Of the diseases favoring the development of tetany, rickets stands first. Rachitic pups are more quickly affected by parathyroid removal than normal pups, and tetany is induced following the removal of a smaller amount of parathyroid tissue than in normal pups.<sup>1</sup>

<sup>1</sup> Pups almost invariably become rachitic when fed a diet of bread, liver, lung, and heart muscle, although if crushed bone is added it is prevented. Similar observations have been made with lion cubs at the London Zoölogical Gardens (17).

The close relationship between infantile tetany (carpopedal spasm) and rickets has long been known and many observers believe that this form of tetany is identical with true parathyroid tetany. Yanase (18) reported the finding of hemorrhages in the parathyroids in a significant number of cases of tetany in rachitic infants and believed that a direct relationship existed, but Oppenheimer (19), Bliss (20), Jörgensen (21), and others could not confirm this finding. There could be a grave functional insufficiency without any gross lesions. If the parathyroids were affected we should rather expect an hypertrophy or hyperplasia as a more frequent sign of insufficiency than the presence of atrophic changes, just as occurs in the thyroid. Slight degrees of parathyroid hypertrophy are extremely difficult of proof, as the mammalian parathyroid does not appear capable of a striking overgrowth. Erdheim (22), Bauer (23), and Strada (24) have described hypertrophic changes in the parathyroids in osteomalacia in man. In rickets and infantile tetany it is certain that no marked overgrowth of the parathyroids occurs and calcium does not usually affect this form of tetany. However, that some important connection exists between rickets and infantile tetany and between rickets and the ease and rapidity of onset of true parathyroid tetany in dogs seems established. How much may be due directly to the parathyroids and how much is due to reducing the factors of safety, one of which is certainly calcium, remains unknown.

#### RELATION OF THE PARATHYROID GLANDS TO SUGAR TOLERANCE.

Dogs are not favorable animals for this work because it is practically impossible to remove the thyroid lobes without destroying the IVth and seriously injuring the IIId parathyroids. Most of the discordant results reported in the literature are attributable to this factor, a notable illustration being Hirsch's (25) observations, when in 1906 as a result of experiments with six thyroidectomized dogs he concluded that the thyroids exercise an important control over carbohydrate metabolism; and two years later, as a result of experiments on fourteen dogs, he concluded that it was not the thyroid but the parathyroid extirpation that produced glycosuria and lowered

the sugar tolerance. He found the glycosuria and hyperglycemia parallel.

Underhill and Hilditch (26), in 1909, as a result of experiments of thyroparathyroidectomy and partial thyroparathyroidectomy showed that sugar tolerance was measurably reduced when three parathyroids were removed, but that two intact parathyroids could maintain normal control over sugar tolerance. Our observations confirm the above findings in that they show that it is not the loss of the thyroid but of the parathyroids that so quickly lowers sugar tolerance. Blood sugar estimations were not made on any of the cases.

The plan followed was to obtain the average amount of glucose per kilo of body weight that each dog could take without alimentary glycosuria, and then to repeat the observations after parathyroidectomy. The technical routine was as follows: Urine was withdrawn with a catheter, and glucose solution containing 20 gm. of glucose per 100 c.c. of distilled water, to which 0.2 per cent. hydrochloric acid was added, was given by stomach tube. The dogs were catheterized in one and two hours. All dogs were on the regular house diet, fed once daily in the afternoon, and sugar tolerance estimations were made in the morning, an interval of at least three days, and in most cases more, being allowed between observations. The Worm-Müller, Nylander, and Benedict qualitative tests were used with each specimen.

Ten dogs were thus observed and the results are given in table I.

TABLE I.

Dog No.	Before parathyroidectomy.		After onset of tetany.		Presence of sugar in urine unrelated to its administration.	Result.
	No. of observations.	Average tolerance in gm. per kilo.	No. of observations.	Average tolerance in gm. per kilo.		
A-167	4	8 to 9			On 11th day	Fatal.
A-183	4	9 to 10	8	2 to 3		Not fatal.
A-208	4	8	9	2 to 3	Negative	Fatal.
A-210	2	7 to 8			On 5th day	Fatal.
A-213	4	6 to 7	3	2 to 3	Negative	Fatal.
A-217	20	2 to 3	5	2 to 3	On 3d day	Not fatal.
A-212	5	6 to 7	1	4	Negative	Not fatal.
A-214	3	9	3	2	Negative	Not fatal.
A-226	13	5 to 6	6	4 to 5	Negative	Not fatal.
A-211	6	2 to 3	21	2 to 3	On 3d day	Not fatal.

It is seen from the above table that in adult bitches great differences (varying from 9-10 to 2-3 grams per kilo) occur in the natural tolerance from glucose. This must be determined separately, therefore, for each dog. In eight of the ten cases a considerable fall was noticed after parathyroidectomy, while in two with naturally low assimilation limits the tolerance remained unchanged after the removal of at least three parathyroids. The feature of most interest is that parathyroidectomy does not result in constant glycosuria. MacCallum and Voegtl (27) reported constantly negative results. Such dogs on the average are capable of assimilating from two to three grams per kilo without its appearance in the urine, and more than this quantity is occasionally not tolerated by dogs with intact parathyroids. This suggests that the parathyroids are only a part of a system controlling sugar metabolism. The thyroids were in all these cases slightly goitrous and, for the most part, removed along with the parathyroids, but from data to be published later regarding thyroid and sugar tolerance we have concluded that the presence or the absence of the thyroid has little or nothing to do with this acute change in alimentary sugar tolerance. The administration of calcium salts does not seem to modify the altered sugar tolerance of parathyroidectomy.

#### CALCIUM SALTS IN TETANY.

Calcium salts are palliative and preventative, but in no sense curative. MacCallum and Voegtl (27) have shown that in nearly all cases of parathyroid tetany calcium salts introduced by mouth or intravenously quickly but temporarily relieve the tetany. In this series there was one case where large quantities of calcium chloride by mouth failed to modify the symptoms, and later intravenous injection of considerable quantities likewise failed to modify the symptoms materially. Such cases must be exceedingly rare, however. After total parathyroidectomy calcium salts tend to, and in most cases do, prolong life. The mechanism of its action is unknown. There were several cases in the series that lived 8, 10, 15, and even 20 days with the aid of calcium salts, that as experience has shown usually die in a few hours or a few

days. On the other hand our observations have shown that in cases where a remnant of active parathyroid tissue remains, calcium salts will permanently save such animals which otherwise would succumb to violent tetany. In our series of thirty-eight, four (A-121, A-148, A-183, and A-217) were surely saved by this means. Two others that died of fatal tetany (A-104 and A-115) could in all probability have been saved by the use of calcium salts for a few days as compressed but not necrotic parathyroids were found at the superior pole at autopsy. In tiding over otherwise fatal tetanies, therefore, calcium salts are of the greatest value. This fact is of clinical importance in operations on goitre in man. Total destruction of the parathyroids in the operation for human goitre should not occur except in the most extensive dissections for old complicated goitres and tumors, while partial destruction (the IVth on the given side) normally occurs when lobectomy is performed. The destruction of one or even two under normal conditions in man or dogs produces no untoward effects. When tetanies develop following operations calcium salts are of the greatest value in tiding over acute insufficiencies. The use of calcium is also of value in testing the completeness or incompleteness of the parathyroidectomy. If the operation is complete calcium will not permanently prolong life. As an example of the effect of calcium in prolonging life the following experiment is reported.

*Dog A-121.*—Middle-aged fox terrier; male. Apr. 20, 1908. Weight 7.3 kilos. Following the eleventh thyroid operation on Dec. 22, although 1 c.c. of saturated aqueous solution of calcium chloride was given just after the operation and another 1 c.c. dose on the following morning, he developed mild signs of tetany about noon and in the afternoon the dose was doubled. Dec. 24. Legs spastic, but the animal eats heartily and is able to move about. Weight 11 kilos. Dec. 27. Slight tetany. Dec. 29. Quite marked tetany; very ataxic. 3 c.c. saturated calcium chloride solution. Dec. 31. Slight twitching. Jan. 1, 1909. Well marked tetany; calcium chloride given. Jan. 2. Severe tetany; unable to stand; changed to lactate and given 10 c.c. of 5 per cent. solution daily. Slight signs of tetany each day occasionally reaching severe tetany until Jan. 8; then the dose was increased to 20 c.c. daily. Weight 10 kilos. Eats heartily. Exposure to cold appears to bring on tetany. Jan. 11. Slight tetany. Jan. 14. Marked tetany. Respiration 88. Pulse 180. Abdomen distended. Nose, ears, and feet hot. Jan. 15. A canine parathyroid was transplanted into the right rectus muscle. No calcium was given until Jan. 24, when slight signs of tetany appeared again. Calcium lactate was started again. Jan. 25. Slight tetany. Jan. 26. Violent tetany. Jan. 29.

Violent tetany. Feb. 1. Slight sign of tetany. Feb. 2. Tetany. 10 ox parathyroids given by mouth. Feb. 3. 30 parathyroids given by mouth; definite twitching. Feb. 4. 100 parathyroids given by mouth; twitchings; and in the afternoon tetany became severe and calcium chloride was given. Weight 10.4 kilos. Feb. 6. Slight twitchings relieved by calcium chloride. Feb. 8. Tetany. 50 c.c. of 5 per cent. solution of calcium lactate given. Feb. 9. Under ether one and one half sterile ox parathyroids were inserted into the right rectus muscle, 25 c.c. of calcium lactate being given at the end of the operation. Feb. 10, 11, and 12. Free from tetany. Feb. 13. Tetany. Evidence of gastric irritation, vomits frequently, considerable amounts of mucus. Given 50 c.c. of 0.5 per cent. solution of calcium chloride subcutaneously. Feb. 15. Tetany. Respiration 180. Pulse 210. Apparently suffering pain. Calcium lactate given in milk. Feb. 16. Mild tetany. It appears evident now that calcium salts are not well borne when administered by mouth. Feb. 18. Free from tetany. Calcium lactate given in milk. Feb. 19. The fourteenth operation for thyroid removal; much scar tissue about the remaining stump. The animal rallied quickly, ate heartily, and was given 50 c.c. of 5 per cent. solution of calcium lactate in milk about 6 P. M. Feb. 20, 8 A. M. Dead from hemorrhage.

*Autopsy.*—The hemorrhage is seen to be from the superior thyroid artery which had been cut almost flush with the carotid trunk, and the stump was too short to hold the ligature. Viscera are blanched. Two accessory thyroids, the size of small cherries, were found imbedded in the thymus. Chronic catarrhal gastritis, probably from calcium salts. The other tissues appeared normal. At the time of operation the dog seemed to be in perfect health, and was fat and sleek.

As regards the administration of calcium, the stomach route is preferable to all others and is equally efficient, since the few minutes' quicker action when given intravenously is offset by the greater dangers. Subcutaneous injection is also not to be recommended on account of the irritant action, discomfort, and danger of necrosis. In all our dogs gastric irritation was present. This could have been much lessened by giving the lactate of calcium in milk instead of the highly irritant calcium chloride which is more soluble and acts more quickly. Whether the daily use of calcium as bone would save such dogs is not known. Our few experiences with the temporary use of bone indicate that it also aids.

#### PARATHYROID OPOTHERAPY.

Unlike thyroid feeding in myxedema, the feeding of parathyroid glands by mouth has no influence over the course of tetany. MacCallum and Voegtlind (27) reported negative results from their experiments, and we have given as high as 100 fresh ox parathyroids daily to dogs with complete tetany without any amelioration of symptoms or prolongation of life. In another case

(dog A-121) of incomplete but permanent tetany, ten ox parathyroids were given on one day, thirty on the following day, and 100 on the third day, also with negative results. In the same dog one canine parathyroid taken aseptically and inserted into the right rectus muscle maintained the dog free from tetany for eight days, and at the end of this period histological examination of the scar showed no evidence of the parathyroid. A large ox parathyroid was then inserted and this likewise relieved the tetany for five days. MacCallum (27) refers to an observation of Halsted's where feeding ox parathyroids by mouth seemed of value in a case of human tetany, although Shepard (28) reports negative results from a similar experience. MacCallum (29) and others have shown the rapid relief of symptoms by the intravenous injection of fresh parathyroid emulsions, and Beebe (30) has further shown that the parathyroid nucleoproteids also relieve tetany when introduced intravenously.

Transplantation is a difficult procedure and although there are many reports of successes the data of permanency are often incomplete. Halsted (31) points out the necessity of first creating a physiological insufficiency, but even then most of the attempts are failures. Our three attempts to transplant them into the rectus muscle were failures, although the dogs were temporarily relieved of tetany from five to eight days. Bone marrow, spleen, and thyroid are said to be more favorable sites for transplantation.

#### SUMMARY.

Accessory parathyroid tissue unassociated with the thyroid lobes is present in 5 to 6 per cent. of dogs. For the determination of the presence of accessory parathyroid tissue there are two tests: (1) the anatomical, often entailing serial sections of the neck and upper thoracic structures, and (2) the biological, consisting of the daily use of some calcium salt for two to three weeks. The latter is more easily carried out and more accurate. In the absence of all parathyroid tissue calcium salts will not save the animal's life, while in the presence of active parathyroid tissue calcium will save it. Many factors other than the amount of parathyroid tissue removed influence the onset of tetany, among which are age, pregnancy, lactation, rachitis, the administration of sulphur, and diet.

Pregnancy and lactation tetany in dogs resembles in all essentials parathyroid tetany. Our observations are in harmony with those who hold that the thyroid and parathyroid are independent structures as regards their anatomy, physiology, and pathology. The removal of the parathyroids immediately lowers alimentary sugar tolerance, but rarely to the degree of constant glycosuria.

In sharp contrast with thyroid substance in myxedema, the feeding of parathyroid substance fresh or dried by mouth is of no value in the parathyroid tetanies of dogs.

Calcium salts have a striking palliative effect on parathyroid tetany and preventative action in tiding over otherwise fatal cases, but are in no sense curative. The mode of action is unknown. There is some evidence that calcium salts directly influence the parathyroid gland.

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## EXPERIMENTAL HYDROCEPHALUS.\*

By WALTER S. THOMAS, M.D.

(From the Department of Pathology of the Washington University Medical School, St. Louis.)

PLATES 18 TO 21.

Many observations indicate that obstruction to the free communication between the ventricular system of the brain and the subarachnoid space may cause internal hydrocephalus. The obstruction with hydrocephalus has been found either at the foramen of Magendie or in the aqueduct of Sylvius or, in some instances of unilateral hydrocephalus, at the foramen of Monro.

Cases of hydrocephalus following cerebrospinal meningitis in which there was occlusion of the foramen of Magendie have been reported by Quincke (1), Baskett (2), Bramwell (3), and others. Neurath (4) reported a case following scarlet fever in which there was a leptomeningitis with obstruction to the foramen of Magendie. Spiller (5) describes a case of hydrocephalus in which the aqueduct of Sylvius was completely occluded by a proliferation of neuroglia; in the same communication he describes a case of unilateral hydrocephalus in which there was occlusion of the foramen of Monro on the same side by a mass of tissue which he thought was of a tuberculous nature. Schlapp and Gere (6) have reported two cases of internal hydrocephalus in which the aqueduct was closed by proliferation of neuroglia. Barnes (7) reported a case in which there was a tubercle in the roof of the fourth ventricle completely blocking the foramen of Magendie. Merkel (8) and Goldstein (9) have reported cases of hydrocephalus in which blocking of the outflow of fluid was brought about by cysticerci within the aqueduct of Sylvius. Tumors not infrequently cause closure of the aqueduct and are accompanied by hydrocephalus; cases of this kind have been reported by Quincke (1) and Bonhoeffer (10).

Within the past year three cases of brain tumor in which the aqueduct was closed have come to autopsy at the Washington University Hospital. In all of them there was a well marked dilatation of the lateral ventricles.

Although the clinical and pathological aspects of internal hydrocephalus have been the subject of much study, no effort has been made to explain the pathogenesis of the condition by experiment.

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In the literature I found only one instance in which an attempt was made to reproduce the lesion experimentally.

Burr and McCarthy (11) report a case that clinically resembled meningitis, but at autopsy no meningitis was found. There was a moderate degree of internal hydrocephalus with proliferation of the glia and ependymal cells in the lateral ventricles, proliferation of the interstitial tissue in the choroid plexus with partial encapsulation of the plexus, hyaloid bodies, and lymphoid cell infiltration. There was infiltration of the perivascular lymph spaces about the blood vessels beneath the ependyma with lymphoid cells, and dilatation and congestion of these vessels. From the fact that the changes were limited to the ventricles they thought it possible that a toxic substance in the cerebrospinal fluid might have caused the reaction. In order to prove this view they injected toxic substances, such as urine and tuberculin, into the lateral ventricles of kittens. The reaction in the ependyma, the swelling of the glia, and the round cell infiltration of the blood vessels were quite similar to the changes observed in the case described. There was, however, no dilatation of the ventricles.

The following experiments were undertaken to determine if it is possible to cause an obstruction to the outflow of the fluid by plugging the aqueduct or foramen of Magendie and to produce hydrocephalus by injecting some insoluble substance into the ventricle. Aleuronat was chosen for this purpose; it is a finely granular substance insoluble in the body fluids and produces a well marked inflammatory reaction. Dogs were used in all the experiments.

#### TECHNIQUE.

The dog was etherized, the head shaved, and a longitudinal incision was made in the median line over the parietal bones. The temporal muscle was cut across near its origin and separated from the surface of the bone. A small button of bone five millimeters in diameter was removed with a trephine at a point about one centimeter behind a line drawn over the vertex of the skull joining the anterior margins of the ears and about two centimeters from the median line. On account of the difference in size of the animals and the shape of the heads it is impossible to lay down a hard and fast rule by which this point may be found. The hemorrhage if excessive was controlled with bone wax. The dura was punctured with a sharp needle or with the point of a knife. For puncturing the ventricle a blunt needle with a lateral opening near the end was used. This needle was inserted slowly in a downward and inward direction

until penetration into the ventricle was indicated by the flow of cerebrospinal fluid. The depth to which the needle must be inserted in order to reach the ventricle varies, of course, with the size of the dog, being as a rule between one and a half and two centimeters. After a few drops of cerebrospinal fluid were allowed to escape, the aleuronat suspension<sup>1</sup> was injected by means of a small Luer syringe connected directly with the needle while it lay in position with its tip in the ventricular cavity. The needle was withdrawn, and the muscle and skin were closed with silk. At the death of the animal the brain was hardened *in situ* by injecting 10 per cent. formalin into the carotid arteries.

For purposes of description the experiments may be divided into groups depending on the length of time the animals lived after the operation. The experiments are described briefly to show the relation between symptoms during life, the gross changes in the ventricles of the brain, and the stage of the inflammatory process affecting the ependyma.<sup>2</sup>

#### GROUP I. EXPERIMENTS LASTING LESS THAN ONE WEEK.

*Dog 6.*—2 c.c. of the aleuronat suspension were injected into the right lateral ventricle. The animal appeared sick the next day, seemed to be in pain, and was unable to stand. On the following day there was some improvement, but it was still unable to stand. It was killed with chloroform at the end of the third day.

*Gross Examination.*—The aleuronat suspension was found in both lateral ventricles; the third and fourth ventricles and the aqueduct of Sylvius were plugged with the aleuronat. There was a small hemorrhage in the optic thalamus on the right side and some blood in the right lateral ventricle. This hemorrhage was undoubtedly due to the insertion of the needle beyond the ventricle. There was no dilatation of the ventricles.

*Microscopic Examination.*—There is an abundance of aleuronat surrounded by polynuclear leucocytes in both lateral, the third and fourth ventricles, and in the aqueduct of Sylvius. The choroid plexus is edematous and is infiltrated

<sup>1</sup> The aleuronat was suspended in starch. It was prepared as follows:

Aleuronat .....	5.0
Starch .....	1.5
Water .....	75.0

The starch was placed in cold water; this was slowly brought to the boiling point and the aleuronat added and thoroughly mixed. The mixture was sterilized in the autoclave.

<sup>2</sup> I am under obligation to Dr. Ernest Sachs for the ophthalmoscopic examinations.

with immense numbers of polynuclear leucocytes. There is some proliferation of the ependymal cells lining the ventricular cavities and a slight swelling of the neuroglia beneath the ependyma. The walls of the blood vessels situated beneath the ependyma and for some distance in the brain substance are infiltrated with polynuclear leucocytes.

*Dog 14.*—0.75 c.c. of the aleuronat suspension was injected into the right lateral ventricle. The dog appeared well until the third day when the head was seen to be hyperextended and the legs spastic. Death occurred on the fifth day.

*Gross Examination.*—There was a small hernia of cerebral tissue through the opening in the dura, which extended to the outer surface of the skull. There was hemorrhage and exudate at the base of the brain and about the medulla and extending into the spinal canal. On section the lateral ventricles were slightly dilated. The foramen of Monro on the right side was closed with aleuronat. Both lateral, and the third and the fourth ventricles were filled with the aleuronat. The aqueduct of Sylvius was clear.

*Microscopic Examination.*—All the ventricles contain a large amount of aleuronat surrounded by polynuclear leucocytes. The ependymal cells are proliferated, and there is an accumulation of lymphoid cells beneath the ependyma; the walls of the blood vessels show the same infiltration with polynuclear leucocytes. In the pia at the base of the brain and on the surface of the pons, medulla, and cord there are large numbers of polynuclear leucocytes; there is a small bit of the aleuronat on the posterior surface of the medulla.

*Dog 26.*—1 c.c. of the aleuronat was injected into the right lateral ventricle. On the second day the dog appeared quite ill; pressure over the scalp was obviously painful. On the third day the animal was unable to stand and there was a paralysis of the left side of the body. On the fourth day there was a definite cellulitis of the scalp. The animal was killed with chloroform at the end of the fourth day.

*Gross Examination.*—On section both lateral ventricles were slightly dilated; they measured 13 by 10 mm. at the level of the infundibulum. Aleuronat could be seen in the right lateral and the third ventricles. The aqueduct was patent.

*Microscopic Examination.*—The right lateral ventricle contains some blood and many polynuclear cells. Polynuclears are seen in the choroid plexus in all the ventricles, and there are a few large mononuclear cells. Small bits of the aleuronat are present in all the ventricles, more in the right lateral ventricle than elsewhere.

In these experiments there is very slight dilatation of the ventricles. In each instance the exudate is composed almost entirely of polynuclear cells. The walls of the blood vessels are infiltrated with the same cells. In dog 14 there was an acute meningitis probably due to the irritation caused by aleuronat which had found its way into the subarachnoid space through the foramen of Magendie, though possibly by an infection. The picture is one of an acute inflammation.

The experiments show that aleuronat injected into the right ven-

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the third, and to a slight extent the fourth. The lateral ventricles at greatest dilatation measured 12 by 6 mm. A small bit of the aleuronat in the descending horn of the left lateral ventricle.

*Optic Examination.*—The floor of the third ventricle shows a proliferation of ependyma and an accumulation of lymphoid cells, plasma cells, large mononuclears, and giant cells. In the aqueduct there is a small bit of the aleuronat imbedded by the same cells, the proliferation of ependyma is well marked. Choroid plexuses in the lateral ventricles are infiltrated with lymphoid cells. The proliferation of the connective tissue; that in the fourth ventricle normal.

—1 c.c. of the aleuronat suspension was injected into the right lateral

The animal behaved normally until the tenth day when it became listless and looked sick; the hind legs were held stiffly in walking, and manipulation of the legs was met with resistance suggestive of muscle spasm; this resistance to motion had increased somewhat on the fifteenth day, and then remained about the same until death on the twentieth day. Evidence that the brain tissue was under pressure was afforded by a hernial protrusion of the cerebellum through the trephine opening. The cerebellum, moreover, projected over the foramen magnum and there was a well defined groove at the point where the bony margin of the foramen was in contact with the cerebellar tissue. On section of the brain at the level of the optic commissure there was a well marked dilatation of the lateral ventricles, the right measuring 6 by 10 mm., the left 5 by 15 mm.; the third and fourth ventricles were not dilated and the aqueduct of Sylvius was patent. No exudate could be seen on gross examination.

The ophthalmoscopic examination made shortly before death showed a pale disc on both sides. Figure 1 is from a normal brain for comparison; figure 2 is from the brain of dog 9.

*Microscopic Examination.*—There is some proliferation of the ependymal lining the ventricles; the choroid plexus (figure 10) in the lateral and third ventricles has aleuronat imbedded in its folds and surrounded by small and large mononuclear cells, connective tissue cells, and a few giant cells. In the fourth ventricle there are a few small mononuclear and polynuclear cells. There is a marked proliferation of the ependymal cells lining the aqueduct of Sylvius (figure 11), and the blood vessels under the ependyma show the same infiltration with small mononuclear cells as that described above.

*Dog 10.*—1 c.c. of the aleuronat suspension was injected into the right lateral ventricle. There was at first some stiffness of the legs and on the sixteenth day there was what appeared to be spastic paraplegia. The eyes were clear but the animal appeared to be blind. It died on the eighteenth day.

*Gross Examination.*—At the site of puncture the cerebral tissue protruded through the trephine opening for a distance of about four millimeters. The cerebellum shows a groove corresponding to the edge of the foramen magnum. On section both lateral ventricles were dilated, the left measuring 8 by 10 mm., the right 5 by 7 mm. The right foramen of Monro was plugged with aleuronat. There was no dilatation of the third ventricle. The fourth ventricle was compressed. The aqueduct of Sylvius was closed.

*Microscopic Examination.*—The choroid plexus in the right lateral ventricle is distorted and thickened; there is a mass composed of connective tissue cells,

large mononuclear cells, lymphoid cells, and some giant cells. In the left lateral ventricle the choroid plexus shows no change except slight edema. In the walls of the right ventricle the walls of the blood vessels are infiltrated with lymphoid cells in considerable numbers (figure 9); this change is not seen to the same extent on the left side. The aqueduct of Sylvius is completely plugged with aleuronat, necrotic tissue, lymphoid cells, and connective tissue cells.

*Dog 16.*—1 c.c. of the aleuronat suspension was injected into the right lateral ventricle. The dog remained well until the twentieth day when it appeared ill; it gradually grew worse and died on the twenty-fifth day.

*Gross Examination.*—Both lateral ventricles were dilated (figure 3); they measured about 10 mm. in diameter at the level of the hypophysis. The third ventricle, the anterior part of the aqueduct of Sylvius, and the fourth ventricle were slightly dilated. The aqueduct was patent throughout. The foramina of Monro appeared to be open.

*Microscopic Examination.*—The choroid plexus in the lateral ventricle is thickened. There is infiltration of lymphoid, large mononuclear, and connective tissue cells. The walls of the blood vessels are infiltrated with lymphoid cells.

*Dog 17.*—0.75 c.c. of aleuronat suspension was injected into the right lateral ventricle. The animal remained well for two weeks, then began to lose weight, became inactive and gradually sank into a comatose condition. Death occurred on the twenty-third day after operation.

*Gross Examination.*—Both lateral ventricles were dilated (figure 4), the right measuring 6 by 4 mm., the left 9 by 10 mm. The aqueduct of Sylvius was patent and apparently slightly dilated; the third ventricle was not dilated, nor was the fourth. The foramen of Monro on the right side was occluded.

*Microscopic Examination.*—The choroid plexus of the lateral ventricle is thickened and infiltrated with small and large mononuclear cells and a few giant cells; there is marked proliferation of the glia beneath the ependyma (figure 12) and of the ependymal cells. No polymonuclear cells are seen except in the fourth ventricle where there are a few in the choroid plexus.

In this group of experiments the signs of acute inflammation have entirely disappeared and there is more marked increase of connective tissue in the choroid plexus; plasma cells occur in considerable number. There is a greater proliferation of ependyma.

The animals remained well during the period from ten to twenty days and displayed symptoms suggesting cerebral pressure. In one instance the occurrence of choked disc was demonstrated. Further evidence of increased intracerebral pressure was furnished by hernial protrusion of the brain substance through the trephine wound and by projection of the cerebellum into the foramen magnum. These changes were explained by well marked internal hydrocephalus found in this group of experiments.

**GROUP IV. EXPERIMENTS OF DURATION LONGER THAN TWENTY-EIGHT DAYS.**

*Dog 19.*—Puppy five weeks old. In puncturing the brain no cerebrospinal fluid was obtainable. 0.5 c.c. of the aleuronat suspension was injected with the needle in the position at which the ventricle was usually found. The dog recovered readily and appeared to be well. On the thirtieth day a second attempt was made. Again it was impossible to obtain cerebrospinal fluid. 0.5 c.c. of the aleuronat was injected. Sixteen days later a third puncture was made, 1 c.c. of cerebrospinal fluid was removed and 0.25 c.c. of aleuronat injected. On the following day the dog was very restless; on the next day he was unable to stand, the eyes were staring, and the head was jerked to the right at short intervals. Both lateral ventricles were tapped, and 1.5 c.c. of fluid were withdrawn from each. Fluid from the right was turbid and contained many polynuclear cells. Fluid from the left was clear and colorless. The next day the animal was much better and was able to walk, the eyes remained staring, and the upper lip contracted spasmodically. Examination of the eyes showed well marked choked disc on both sides. The animal was killed with chloroform thirty days after the last puncture and three months and seven days after the first operation.

*Gross Examination.*—Both lateral ventricles were dilated. The anterior horn of the right lateral ventricle was more widely dilated than that of the left and measured 12 cm. in diameter, while the posterior and descending horns of the left were greater than the right, the greatest diameter being 20 mm. The anterior horn of the right ventricle was divided by thin friable septa into several small compartments filled with a semifluid opaque material. The left ventricle did not have these septa and was filled with colorless fluid. The third ventricle was not dilated.

*Microscopic Examination.*—The choroid plexus on the right side is thickened with connective tissue and there is some proliferation of the ependymal cells. There is some granular detritus in the folds of the choroid plexus and many large mononuclear cells containing a light brown pigment. The ependymal lining of the ventricles consists of a single layer of flattened cells. In the choroid plexus there are a great many large mononuclear cells containing brown pigment granules.

*Dog 20.*—Puppy five weeks old from the same litter as No. 19. On puncture cerebrospinal fluid was not obtained. 0.5 c.c. of the aleuronat suspension was injected into the right side. The animal appeared quite well following the operation. On the thirtieth day after the first operation the ventricle was punctured, some of the fluid withdrawn, and 0.25 c.c. of aleuronat was injected. Seventeen days later 0.25 c.c. was injected into the right ventricle. On the following day the hind legs were rigid, the dog was unable to stand, and the eyes were staring, exhibiting some exophthalmus. Death on the second day following the last operation was fifty-two days after the first.

*Gross Examination.*—There was a small cerebral hernia at the site of puncture. On section the ventricles were greatly dilated (figure 5), the right measuring 8 by 10 mm. at the level of the optic commissure, the left 13 by 18 mm. The greatest dilatation was seen in the descending horns, the left measuring 10 by 13 mm., the right 18 by 13 mm. In the right ventricle there were soft thin

septa dividing the ventricle into several compartments. The third and fourth ventricles were not dilated. The aqueduct of Sylvius was patent throughout.

*Microscopic Examination.*—The right lateral ventricle contains a large quantity of exudate made up chiefly of polynuclear cells. This acute inflammation seems to be superimposed on one of a more chronic character in which the choroid plexus is thickened by connective tissue cells infiltrated with lymphoid and large mononuclear cells. The blood vessels in the walls of the right ventricle are infiltrated with lymphoid cells, large mononuclear cells, and in places with polynuclears. The choroid plexus in the third and left lateral ventricles appears normal; that in the right lateral ventricle is thickened by connective tissue. The ependymal cells have practically disappeared and there are a great many polynuclear cells in the plexus and infiltrating the walls of the blood vessels in the walls of the ventricles. The proliferation of ependyma is well shown in the aqueduct of Sylvius where there is also an apparent increase in neuroglia. The choroid plexus in the fourth ventricle is somewhat edematous.

*Dog 23.*—Puppy from the same litter as group II. The animal died thirty-eight days after operation.

*Gross Examination.*—Both ventricles were dilated, the right slightly more than the left; it measures 8 by 10 mm. at the level of the optic commissure. In the posterior horn of the right lateral ventricle there was a cavity in the brain substance 12 mm. in diameter, extending to within 2 mm. of the cortex; this was connected with the ventricle by an opening about 7 mm. in diameter. It was presumably the site of puncture and the aleuronat, doubtless injected into the brain substance, by destruction of the brain tissue gained entrance to the ventricle. The cavity contained a small amount of semifluid pus-like material. The third and fourth ventricles were not dilated. The aqueduct of Sylvius was patent.

*Microscopic Examination.*—A section through the wall of the cavity shows the picture of brain softening; there is a loose network containing degenerated cells and many compound granular corpuscles, some of which contain brown pigment granules. The wall of the ventricle is infiltrated with lymphoid cells. The choroid plexus is infiltrated with lymphoid and large mononuclear cells and in places with small hemorrhages surrounded by polynuclear cells.

*Dog 38.*—Puppy two days old. A few drops of aleuronat were injected into the right hemisphere in the region of the ventricle. Death occurred sixty days after the operation.

*Gross Examination.*—The right ventricle showed enormous dilatation (figure 6), measuring 18 by 20 mm. in diameter at the level of the hypophysis. The left ventricle showed no dilatation. The aqueduct of Sylvius was patent. There was no dilatation of the third or fourth ventricles.

*Microscopic Examination.*—A section of the wall of the right ventricle shows slight infiltration with lymphoid cells and the neuroglia is somewhat increased. There is no proliferation of the ependyma except in the aqueduct.

All the dogs in this group were very young puppies. In none of them was it certain at the time of operation that the ventricle was punctured. In this group the animals lived more than four weeks and the dilatation of the ventricles is considerable. The

microscopic changes differ very little from those found in the preceding group. In No. 20 there is an acute inflammation following the last injection which was made only two days before death.

In the experiments of group I in one instance (dog 6) the aqueduct of Sylvius, and in another the foramen of Monro was plugged with aleuronat. In group II in every instance with well marked dilatation the right foramen of Monro was plugged. In group III in dog 10 the aqueduct of Sylvius was completely plugged with aleuronat and newly formed tissue and there was well marked dilatation of the ventricles. In dog 17 the foramen of Monro on the right side was plugged. It has not been possible to determine with certainty the condition of the foramen of Magendie, but proliferation of tissue in the roof of the fourth ventricle and elsewhere has suggested the possibility of occlusion by newly formed tissues. A more accurate method of determining the patency of this opening was desirable.

It was thought that the injection of a pigment into the ventricles of dogs with hydrocephalus would make it possible to determine if fluid no longer passed through the foramen of Magendie. For this purpose black India ink was used in a dilution of one part to four parts of water. The following experiment was done on normal animals.

One cubic centimeter of the suspension of the India ink was injected under ether into the left lateral ventricle of three normal dogs (Nos. 32, 33, and 34). They were killed with chloroform at the end of one, one half, and one quarter hours, and the brains hardened *in situ* by formalin injected into the carotid artery. On examination the ink, fixed in position by the formalin, was found in large quantities in the pia arachnoid at the base of the brain, being more abundant around the base of the cerebellum in the region of the foramen of Magendie than elsewhere, but in all three brains the pigment was seen extending over the cerebral hemispheres. There were well defined differences dependent upon the duration of the experiment. Pigmentation was most intense in the dog that had been allowed to live one hour, slightly less in the one living one half hour, and less, but still very distinct, in the third living only one quarter hour after the injection. In the third animal there was

almost no pigment over the upper half of the cerebral hemispheres. The pigment was confined to the subarachnoid space, and on tearing away the pia arachnoid the brain was left quite clean. On section the pigment was seen distributed throughout the ventricular system in all the brains. On microscopic examination the pigment in the ventricles was attached to the ependymal cells, and in no place was it found beneath the ependyma. The experiment offers a method of demonstrating the gradual flow of cerebrospinal fluid from the ventricles of the brain into the subarachnoid space through the foramen of Magendie.

In the following instances the same experiment was carried out on dogs in which hydrocephalus had previously been produced by injection of aleuronat.

*Dog 8.*—1.5 c.c. of the aleuronat suspension were injected into the right lateral ventricle. The animal recovered readily and remained well for four months. Under ether the left side of the skull was trephined and after drawing off about 1 c.c. of cerebrospinal fluid 1 c.c. of the suspension of India ink was slowly injected. One half hour later the dog was killed.

*Gross Examination.*—The surface of the brain is clear and white, none of the ink being visible. On section the lateral ventricles are dilated equally (figure 7) and measure about 12 mm. in diameter. The left lateral ventricle, the third and fourth ventricles, and the aqueduct of Sylvius contain the ink, but there is none in the right lateral ventricle. The foramen of Magendie is readily made out but seems to be closed by a thin web-like membrane.

*Microscopic Examination.*—In sections through the fourth ventricle at the site of the foramen of Magendie there is marked proliferation of ependyma on the floor of the ventricle and a fine network of neuroglia covering the ependyma and containing within the meshes some large mononuclear cells. The roof of the ventricle is composed of choroid plexus arising from each side of the pons and joined in the center by thin strands of connective tissue containing a few small blood vessels. The plexus itself appears edematous, but otherwise is normal. These changes occur at the level of the foramen of Magendie which is apparently closed by the connective tissue.

*Dog 11.*—1 c.c. of the aleuronat suspension was injected into the right lateral ventricle. The dog lived for four months and eight days and was then killed with chloroform, one half hour after injecting 1 c.c. of the diluted India ink into the left lateral ventricle.

*Gross Examination.*—There was a small amount of the ink in the pia at the base of the brain, but much less than that found fifteen minutes after injection of ink into the ventricle of the normal dog already mentioned. There was no evidence of great pressure, no cerebral hernia, nor jamming of the cerebellum into the foramen magnum. On section both lateral ventricles were dilated equally, measuring about 12 by 15 mm. at the level of the hypophysis. In the

posterior horn of the right lateral ventricle was a thin septum of friable tissues. The left lateral ventricle and the third ventricle were filled with ink. There was none in the right ventricle. The anterior end of the aqueduct of Sylvius was very much narrowed, while the posterior end was dilated. The roof of the fourth ventricle was accidentally torn in the examination, making it impossible to determine whether it was closed or not.

*Microscopic Examination.*—There is some proliferation of the neuroglia in the right ventricle. The ventricles are lined by a single layer of flattened cells. In the left ventricle the pigment is seen attached to the lining cells. In the aqueduct of Sylvius there is proliferation of the ependyma and occasionally a small nodule covered by ependyma resembling closely those seen in granular ependymitis.

*Dog 25.*—1 c.c. of aleuronat suspension was injected into the right lateral ventricle. Sixty-eight days after the operation 1 c.c. of India ink was injected into the left lateral ventricle and one half hour later the dog was killed with chloroform.

*Gross Examination.*—There was no ink on the surface of the brain and none at the base. On section both lateral ventricles were dilated; the left measured 10 by 10 mm. at the level of the infundibulum, and the right at the same level was much larger, measuring 22 by 15 mm. The dilatation was more marked in the descending horn but was considerable throughout. The ink was found in the left lateral, the third, and the fourth ventricles, but none was present in the right. The aqueduct of Sylvius was narrowed but patent. The foramen of Magendie was closed by a very thin membrane.

*Microscopic Examination.*—The lateral ventricles are lined by a single layer of flattened ependymal cells. There is some increase in the neuroglia and occasionally there is a small granulation similar to those seen in the aqueduct of Sylvius in the preceding case. In the aqueduct of Sylvius there is some increase in the neuroglia and proliferation of ependyma. The India ink is readily seen in the aqueduct and fourth ventricles. A section through the roof of the fourth ventricle shows that the foramen of Magendie is closed by a thin connective tissue membrane similar to that found in the preceding experiment.

*Dog 37.*—No cerebrospinal fluid could be obtained on puncture, but 0.75 c.c. of the aleuronat was injected into the left cerebral hemisphere with the needle held in the position at which the ventricle was usually found. Sixty-six days afterwards 1 c.c. of the India ink was injected into the left lateral ventricle and a half hour later the animal was killed.

*Gross Examination.*—A small amount of the ink was found at the base of the brain, but there was none on the surface of the cerebrum or cerebellum. On section both lateral ventricles were found moderately dilated. The anterior horns were but little affected and the body of the ventricles at the level of the posterior edge of the hypophysis measured 8 by 8 mm. The posterior horns were moderately dilated. The left lateral ventricle was filled with the ink and there was a very small bit in the right lateral ventricle and in the third and fourth ventricles. A section through the optic thalamus shows a small yellow area in its center.

*Microscopic Examination.*—The ependyma of the left ventricle shows proliferation only at a point where the choroid plexus is attached, and here large

mononuclear cells bearing yellow pigment are seen in the choroid plexus. The lining of the ventricle is covered with the ink. In the optic thalamus is an area in which the brain substance is destroyed and replaced by compound granular cells embedded in a fine network. This is undoubtedly the site of an injury caused by the needle. A section through the wall of the right ventricle shows no abnormality.

Dog 42.—0.75 c.c. of the aleuronat was injected into the right lateral ventricle; forty-seven days later 1 c.c. of the India ink was injected into the left lateral ventricle, and one half hour later the animal was killed.

*Gross Examination.*—The entire surface of the brain was clean, no trace of the ink being seen. On section the ink was found only in the ventricle injected (figure 8). The right lateral ventricle measured 15 mm. in diameter at the level of the infundibulum, while the right measured 10 mm. The descending horns were more widely dilated, the right measuring 20 mm. from above downward.

*Microscopic Examination.*—The right ventricle is lined by a loose network of neuroglia which is apparently edematous; the ependyma occurs as a single layer of flattened cells. The choroid plexus is thin in places and has the appearance of being stretched out; in one area there is nothing but a long double line of flattened cells, and in another area there is merely a line of connective tissue in contact with a dilated vein. The aqueduct of Sylvius is almost obliterated by neuroglia and the ependyma shows marked proliferation.

The experiments with India ink show that obstruction may occur at the foramen of Magendie and at the foramen of Monro. The third and fourth ventricles, surrounded perhaps by more resistant tissue, may exhibit no dilatation. The same condition is found in hydrocephalus in man where the dilatation was brought about by obstruction in the fourth ventricle or the aqueduct of Sylvius; the third ventricle may or may not be dilated.

#### SUMMARY.

Internal hydrocephalus can be produced experimentally by injecting a foreign substance into the ventricles. In these experiments, aleuronat, a granular, insoluble material, has caused an acute inflammatory reaction, characterized in the first week by an exudate consisting largely of polynuclear leucocytes. Later the picture is one of a chronic process; polynuclear cells are replaced by lymphoid and large mononuclear cells and there is proliferation of the connective tissue in the choroid plexus. Proliferation of the ependyma occurs in the first week but becomes more advanced in the second and third weeks, and there is increase in neuroglia more marked in the long continued experiments.

Ordinarily when aleuronat is injected into a serous cavity, the pleural cavity, for example, abundant accumulation of fluid takes place in twenty-four to forty-eight hours, and at the same time polynuclear leucocytes collect. In the experiments in which the irritant was injected into the ventricle of the brain there was little or no dilatation apparent in the first week; absence of dilatation in all probability is due to the free outflow of the fluid. When obstruction occurs during the chronic stage of the inflammatory process dilatation of the ventricle follows. Choked disc and other symptoms of increased intracranial pressure accompany experimental hydrocephalus.

Dilatation occurs slowly and reaches a maximum in about two months. In some of the experiments of longer duration obstruction can be demonstrated in gross or microscopically. Obvious obstruction has not been found by gross examination in all instances, but in the experiments in which India ink was injected into the ventricle before death obstruction to outflow was very readily demonstrated. The third and fourth ventricles were in all instances filled with the pigment, but none appeared on the surface of the brain, whereas in normal dogs the entire surface, especially the base, became deeply pigmented. Obstruction to the circulation of cerebro-spinal fluid causing internal hydrocephalus may occur at the foramen of Monro, in the aqueduct of Sylvius, or, doubtless with greatest frequency, at the foramen of Magendie.

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## EXPLANATION OF PLATES.

## PLATE 18.

FIG. 1. Normal brain.

FIG. 2. Dog 9, group III, showing dilatation of the lateral ventricles.

FIGS. 3 and 4. Dogs 16 and 17, group III, showing dilatation of the lateral ventricles.

## PLATE 19.

FIG. 5. Dog 20, group IV, showing marked dilatation of the lateral ventricles with the septa dividing the right.

FIG. 6. Dog 38, group IV, showing dilatation of the right lateral ventricle.

FIG. 7. Dog 8, showing dilatation of both lateral ventricles and the presence of the India ink in the left lateral and the third ventricle and in the aqueduct of Sylvius.

FIG. 8. Dog 42, showing dilatation of the lateral ventricles and the India ink in the left lateral. The photograph does not show the ink in the third ventricle and the aqueduct of Sylvius; this was plainly seen in the specimen.

## PLATE 20.

FIG. 9. Dog 10, group II, showing the accumulation of lymphoid cells about the blood vessels in the ventricular walls.

FIG. 10. Dog 9, group III, showing thickened choroid plexus.

## PLATE 21.

FIG. 11. Dog 9, group III, showing proliferation of the ependyma in the aqueduct of Sylvius.

FIG. 12. Dog 17, group III, showing proliferation of neuroglia in the wall of the lateral ventricle.



FIG. 1.



FIG. 2.



FIG. 3.

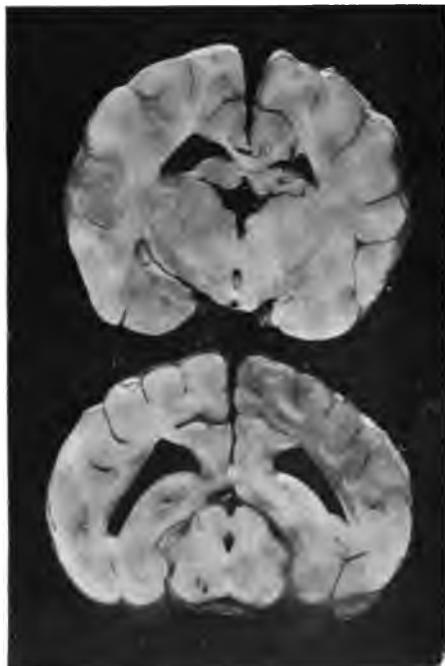


FIG. 4.

Thomas: Experimental Hydrocephalus.

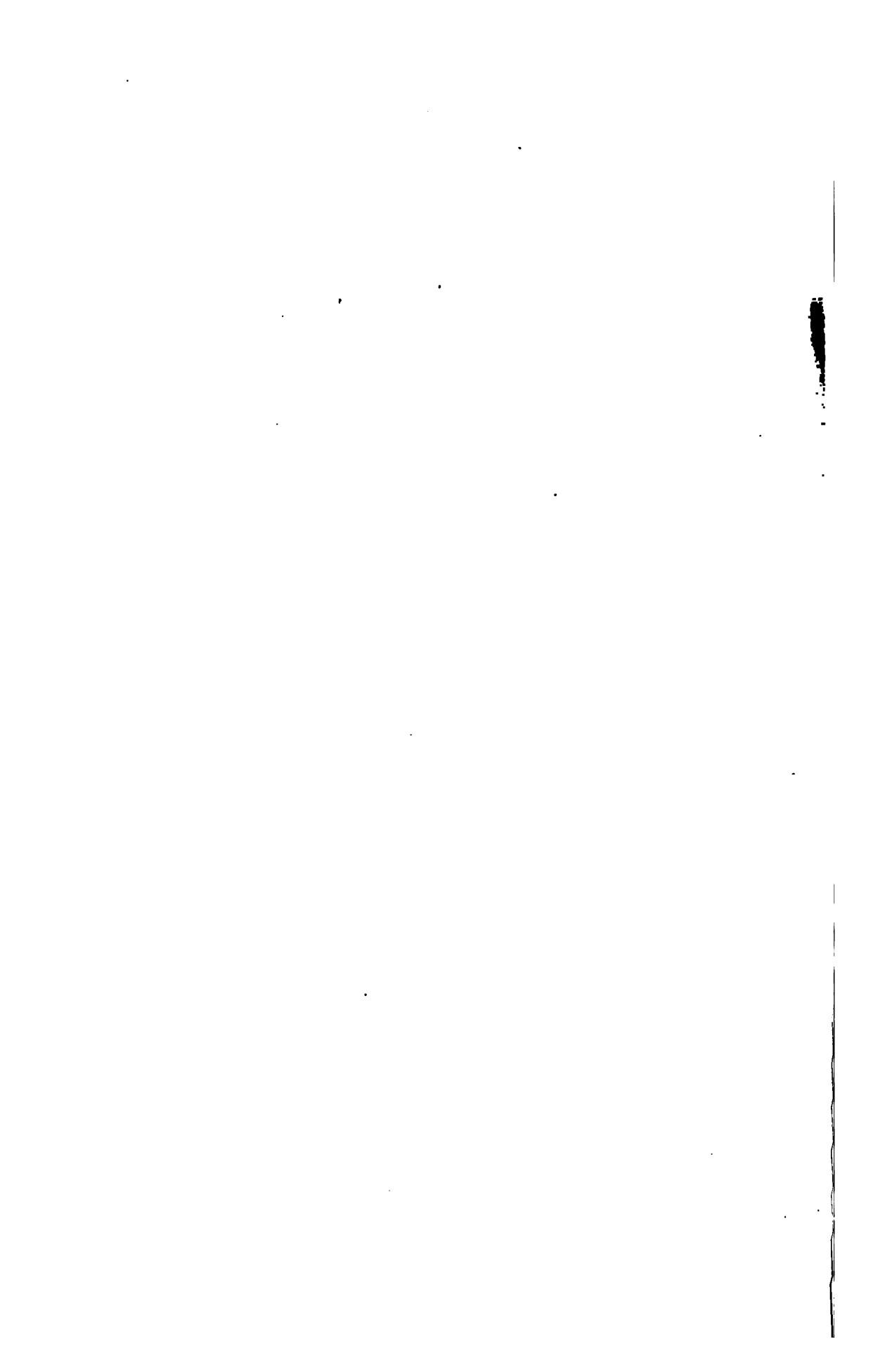




FIG. 5.

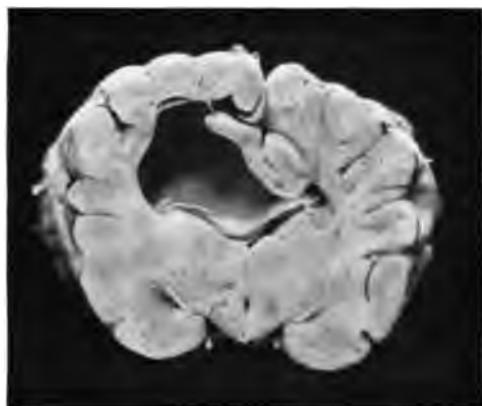


FIG. 6.



FIG. 7



FIG. 8.

(Thomas: Experimental Hydrocephalus.)



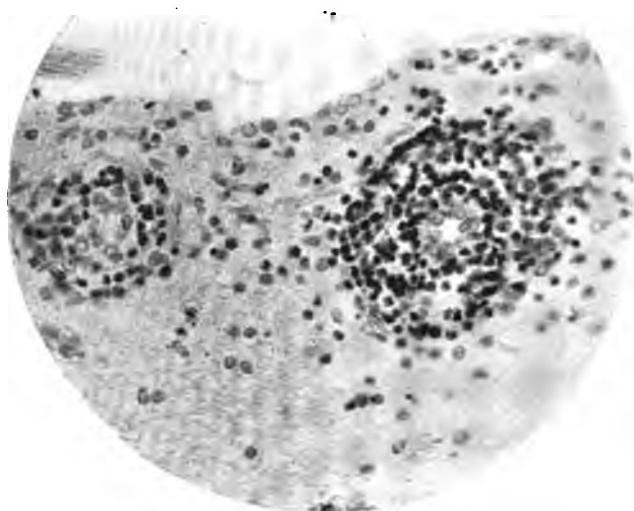


FIG. 9.

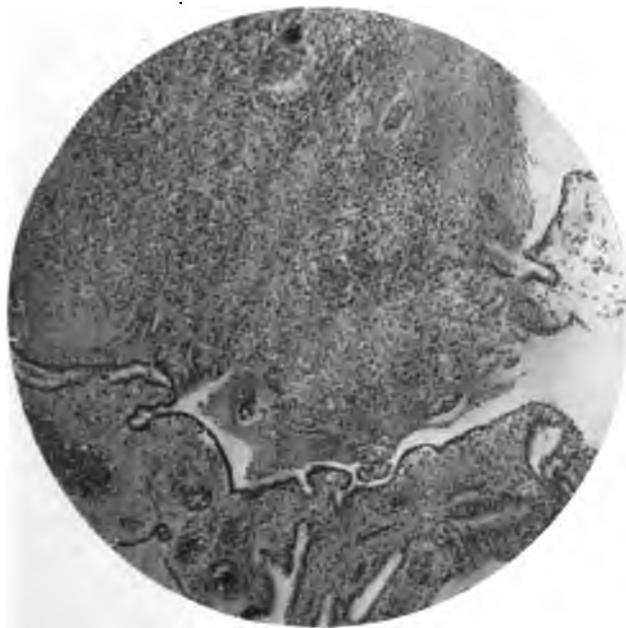
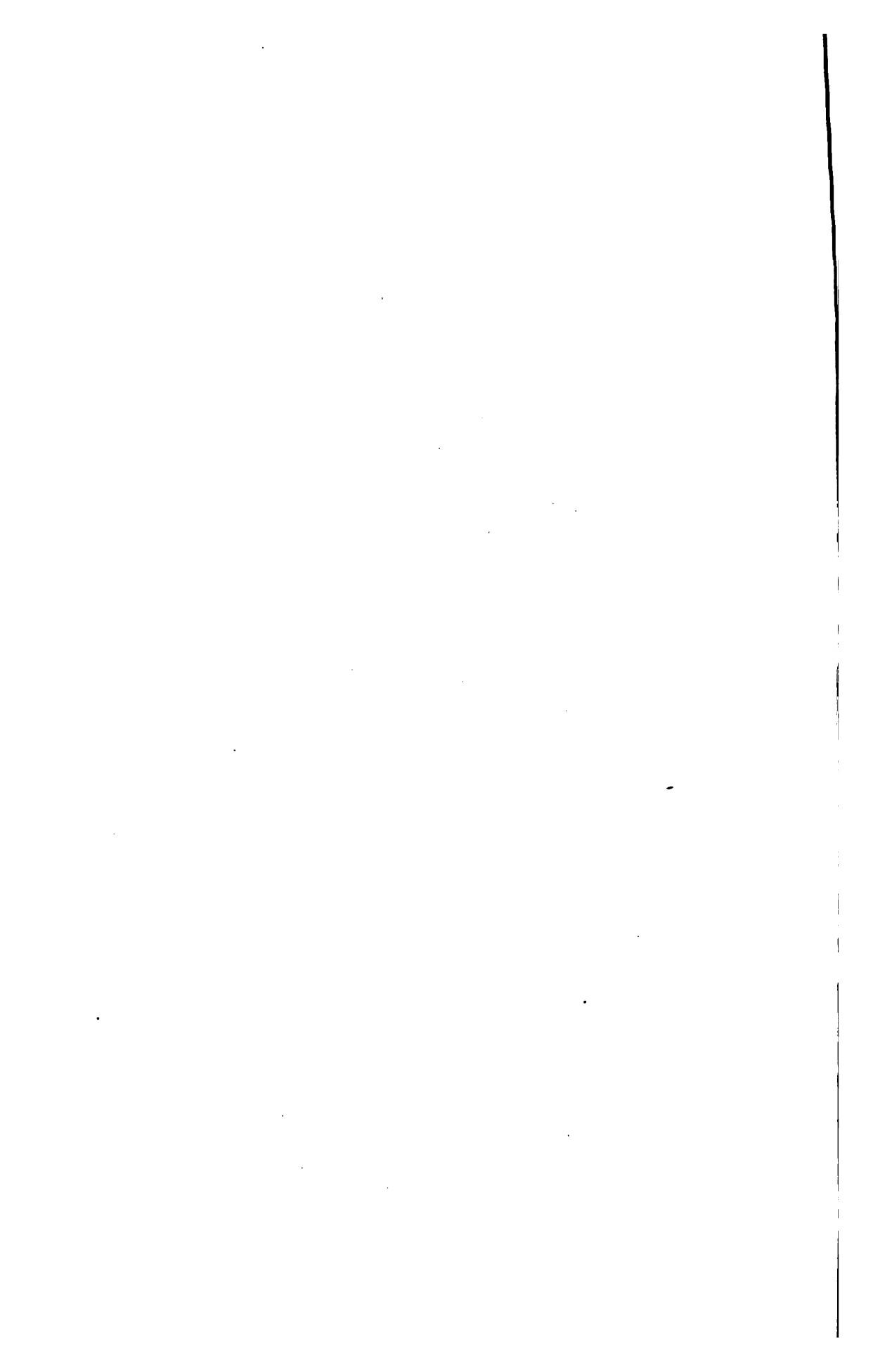


FIG. 10.

(Thomas: Experimental Hydrocephalus.)



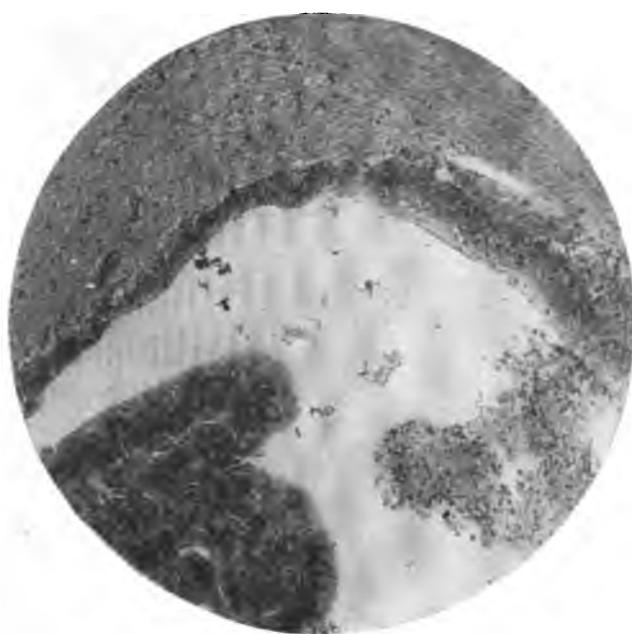


FIG. 11.

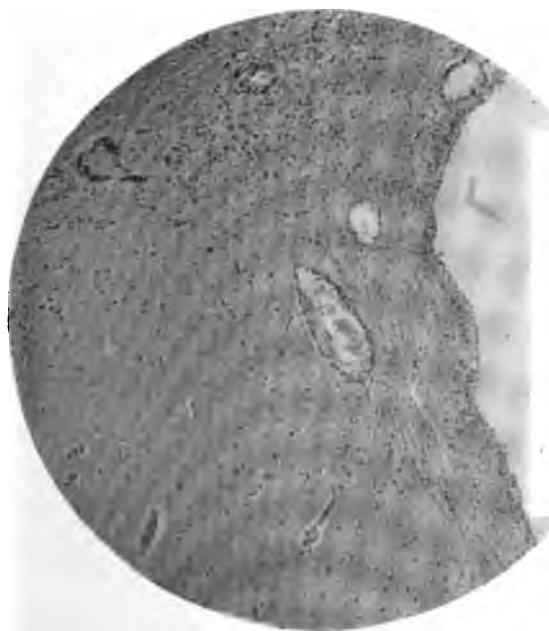
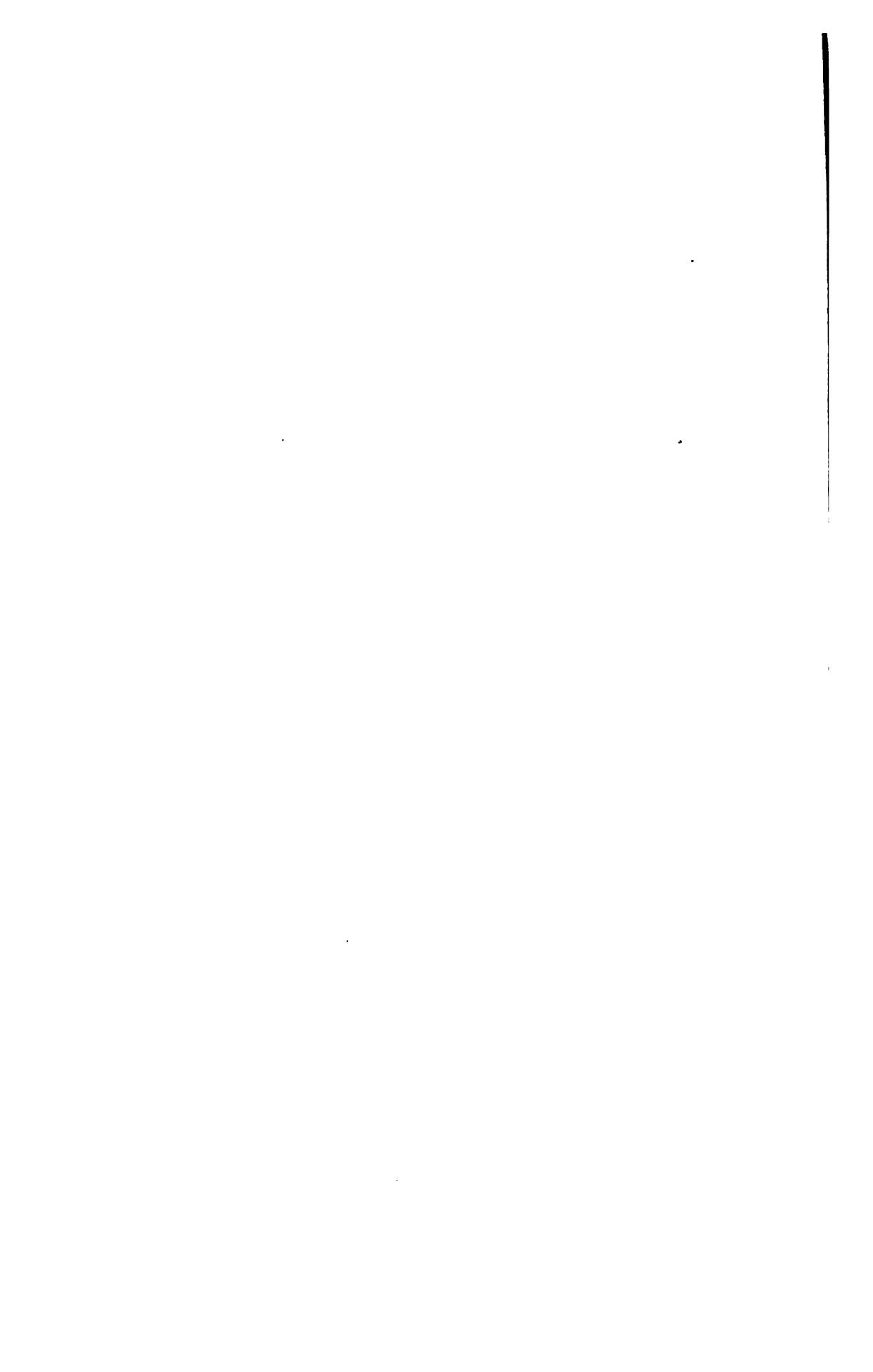


FIG. 12.

(Thomas: Experimental Hydrocephalus.)



# ON THE SURVIVAL AND TRANSPLANTABILITY OF ADULT MAMMALIAN TISSUE IN SIMPLE PLASMA.\*

By ALBERT J. WALTON, M.S.

(*From the Bacteriological Laboratory of the London Hospital, London.*)

PLATES 22 TO 26.

This communication deals with the characteristics of the growth of adult mammalian tissues after transference to fresh medium. In all cases the medium was simple plasma, and the tissues were therefore not stimulated by the addition of tissue extract.

In 1911 Carrel<sup>1</sup> published the results of his experiments on the continued growth of thyroid tissue in simple plasma. At that time he stated that he had not obtained a third generation of cells. Later he and his assistant Ebeling published several papers<sup>2</sup> in which they describe growth persisting for over a year, but in the majority of these cases the subcultures were made in a medium of plasma and tissue extract so that growth was stimulated.<sup>3</sup> Even in such cases Carrel informs me that he has noticed a tendency for the cultures of adult tissues cultivated in media which contain no tissue extract to die; and in a later paper<sup>4</sup> he states that even with embryonic tissue the growth after several passages in normal plasma alone becomes progressively reduced and often stops altogether.

It is necessary that the characteristics of the growth of unstimulated adult tissue be considered before any conclusions can be made as to the effects of various substances upon the rate of growth. The present experiments are therefore concerned only with the

\* Received for publication, October 9, 1913.

<sup>1</sup> Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 416.

<sup>2</sup> Carrel, *idem*, 1912, xv, 516. Ebeling, A. H., *idem*, 1913, xvii, 273.

<sup>3</sup> Carrel, *idem*, 1913, xvii, 14.

<sup>4</sup> Carrel, *idem*, 1913, xviii, 287.



used instead of that of a rabbit. Up to the time of death the cultures had lived for a period of forty-two days.

The tissues of this animal showed after the first transference a marked increase of growth, so that whereas in the first culture growth was present only after an interval of two days, in the second and third subcultures there was usually well marked growth after twenty-four hours. Branching, irregular cells were seen projecting from the edge of the tissue (figure 2), and, if any of the old plasma had been transferred with the tissue, the cells soon passed beyond this into the new medium (figures 1 and 2). Here they rapidly grew and formed an irregular network of cells (figure 3). After a further interval of twenty-four hours the network had become sufficiently dense to form a mass of new tissue resembling the original mass that had been transferred (figure 4). If this mass showed marked increase the tissue was divided before transference. In this way one piece of tissue from this animal was subdivided several times, so that after an interval of twenty-one days there were at the fifth subculture nine pieces of tissue each of the same size as the original piece. At this time growth was such that at the end of the second or third day the ring of new growth was equal in width to the diameter of the original piece of tissue. If a piece of rapidly growing tissue was divided into two it was noticed that the growth was delayed on the cut edge, apparently from the trauma, so that when the cells from the uncut edges were forming a halo of branching cells spreading out into the plasma there were only a few cells projecting from the cut edge. These cells, however, grew rapidly and after the second or third day nearly equalled in width the cells growing from the uncut edge. In the case of the other animals, where growth of the thyroid was not prolonged beyond the third or fourth generation, the rapidity of growth decreased markedly after the first transference. In the first subculture growth was more rapid than in the primary culture, as in the case described above. With the second subculture growth occurred more slowly, being no more rapid than in the case of the first culture, or perhaps even less rapid. At the third transference the cells grew slowly, and if a culture was left for five or six days without transference they became rounded instead of branching, and granular in appearance. In a

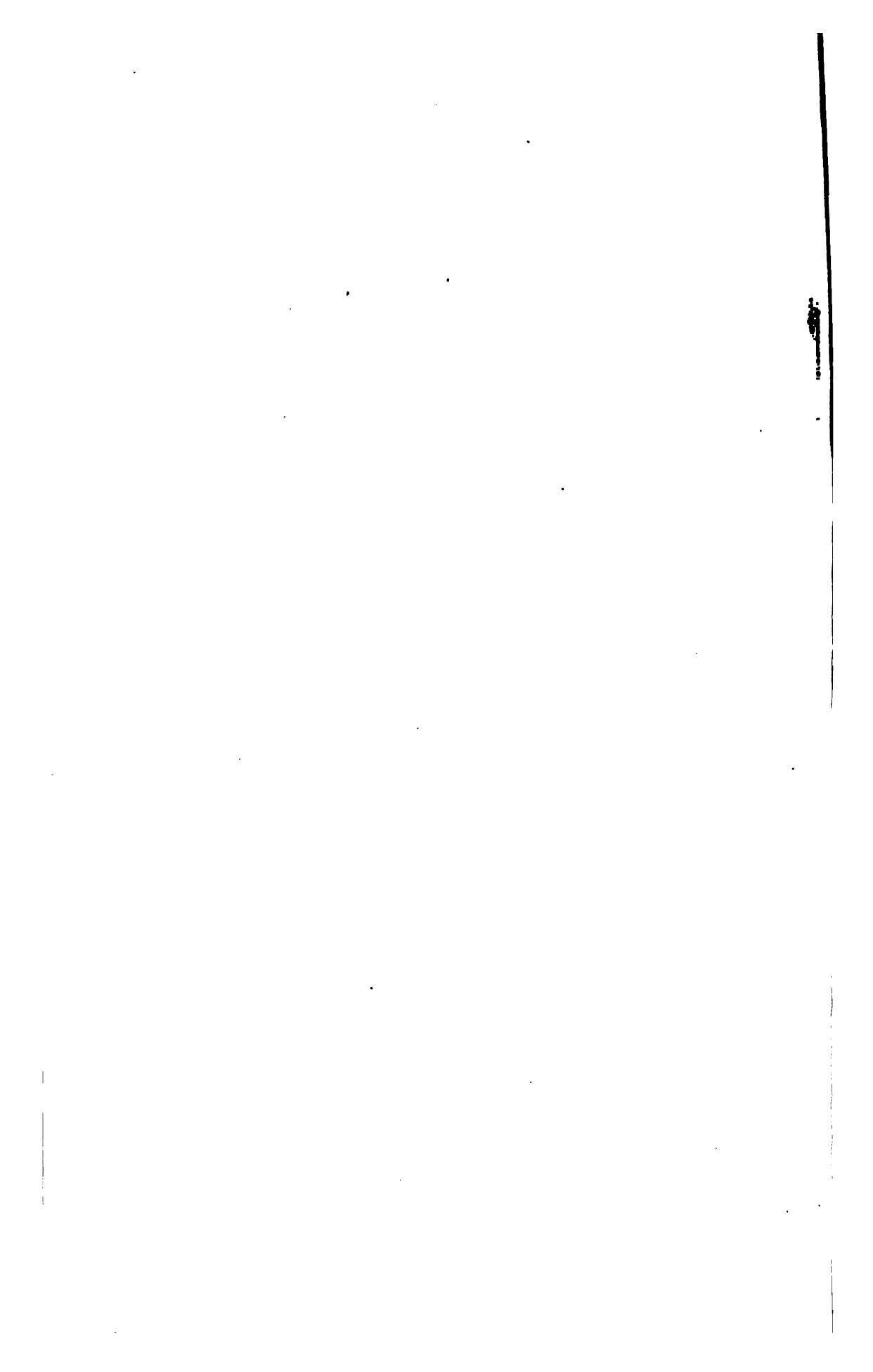




FIG. 5.



FIG. 6.

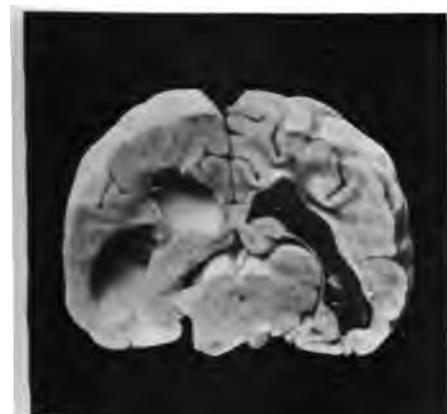
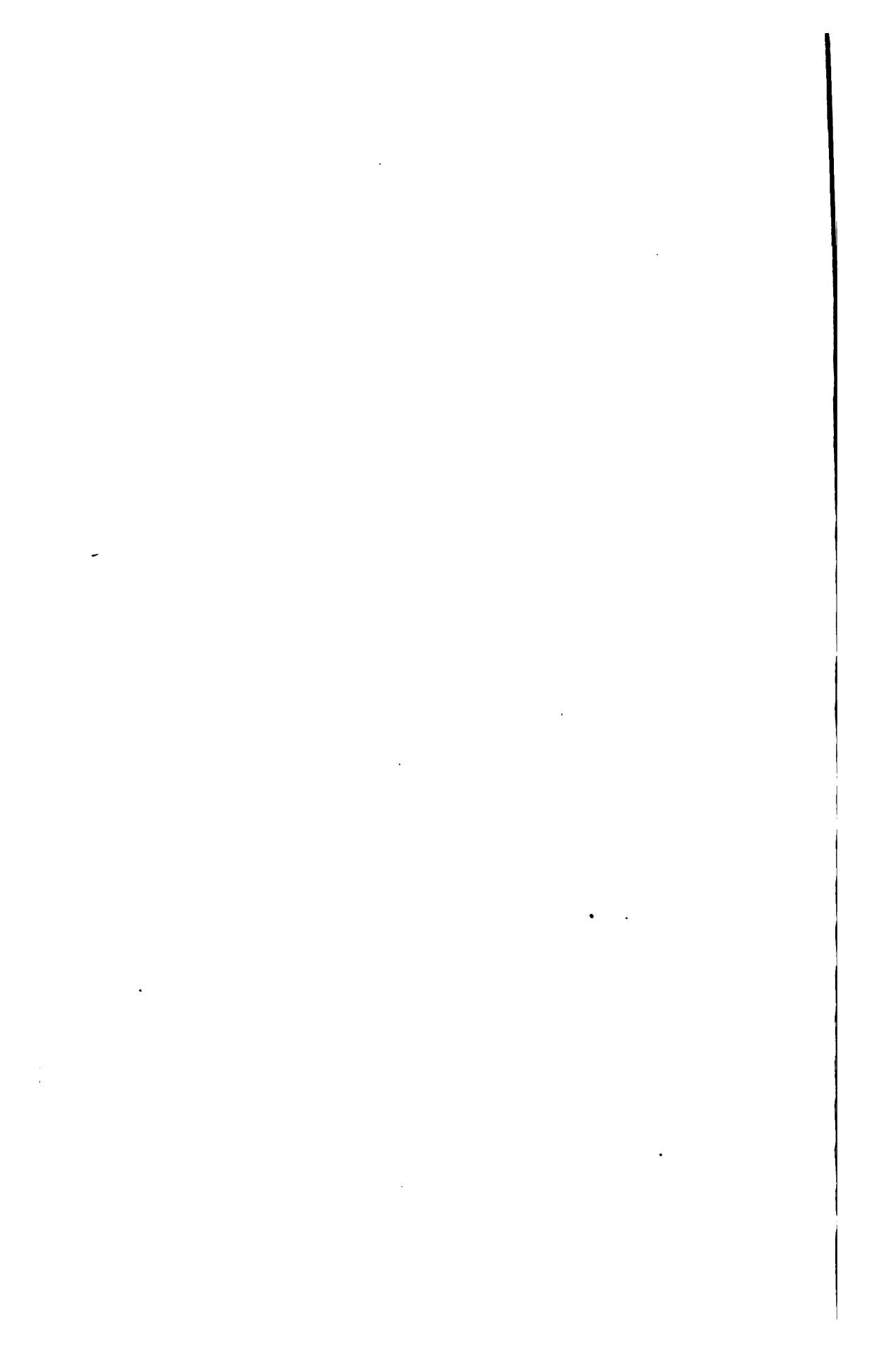


FIG. 7



FIG. 8.

(Thomas: Experimental Hydrocephalus.)



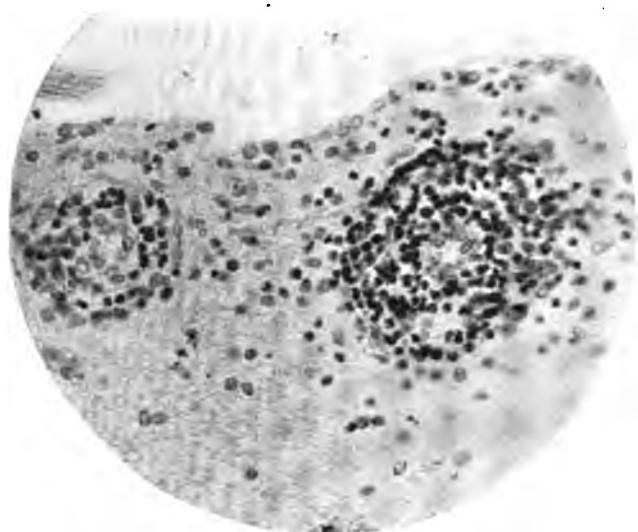
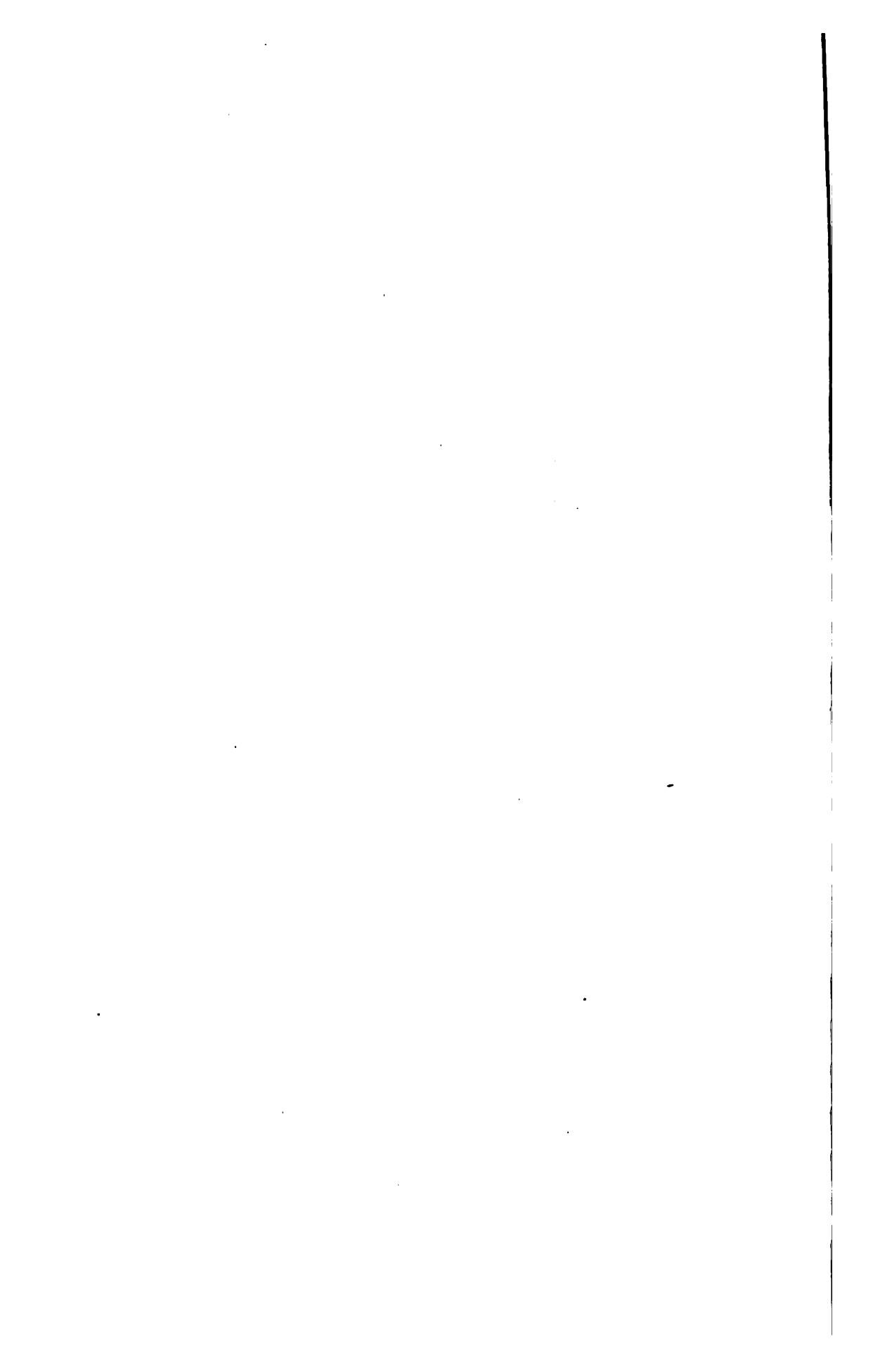


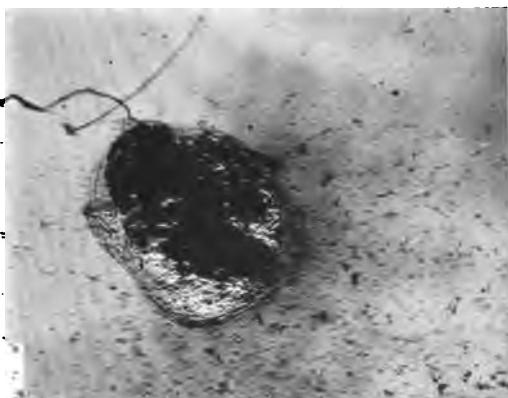
FIG. 9.



FIG. 10.

(Thomas: Experimental Hydrocephalus.)

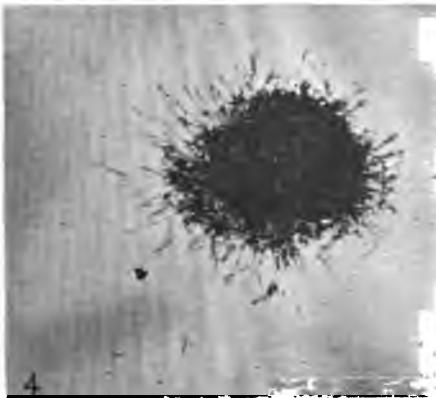




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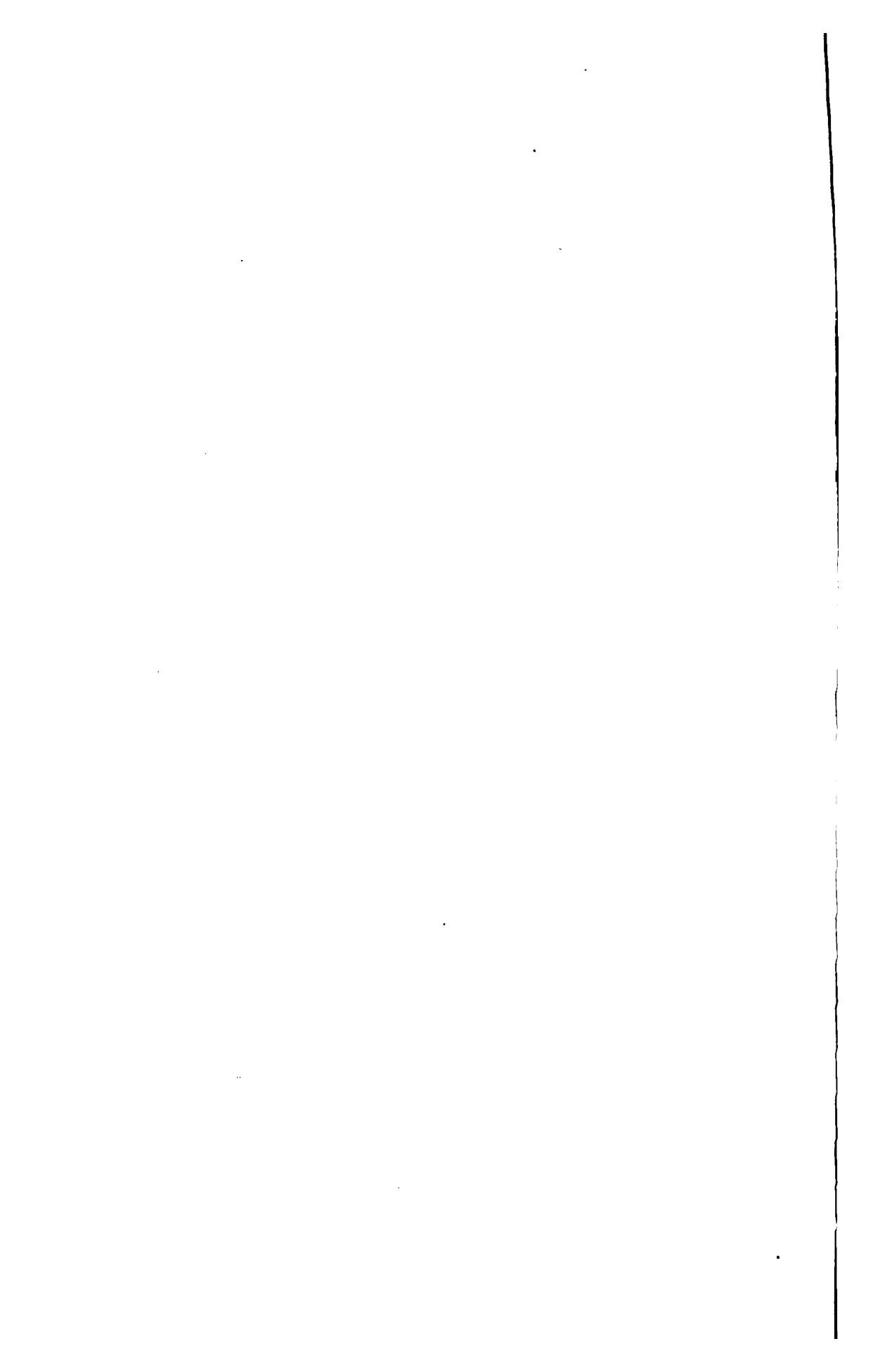


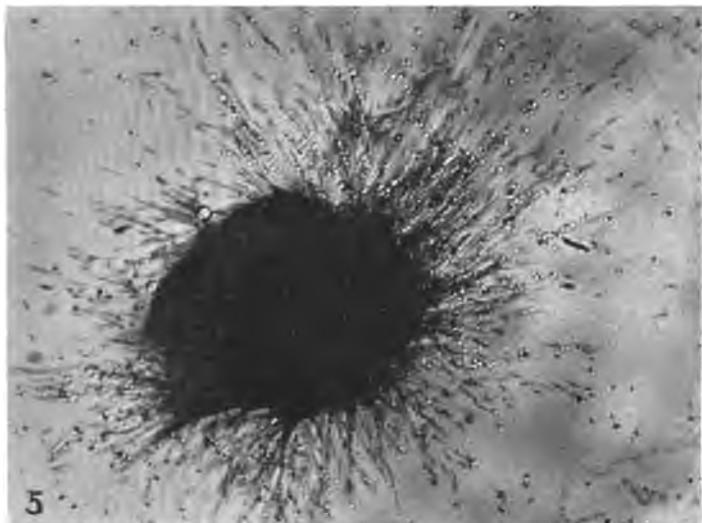
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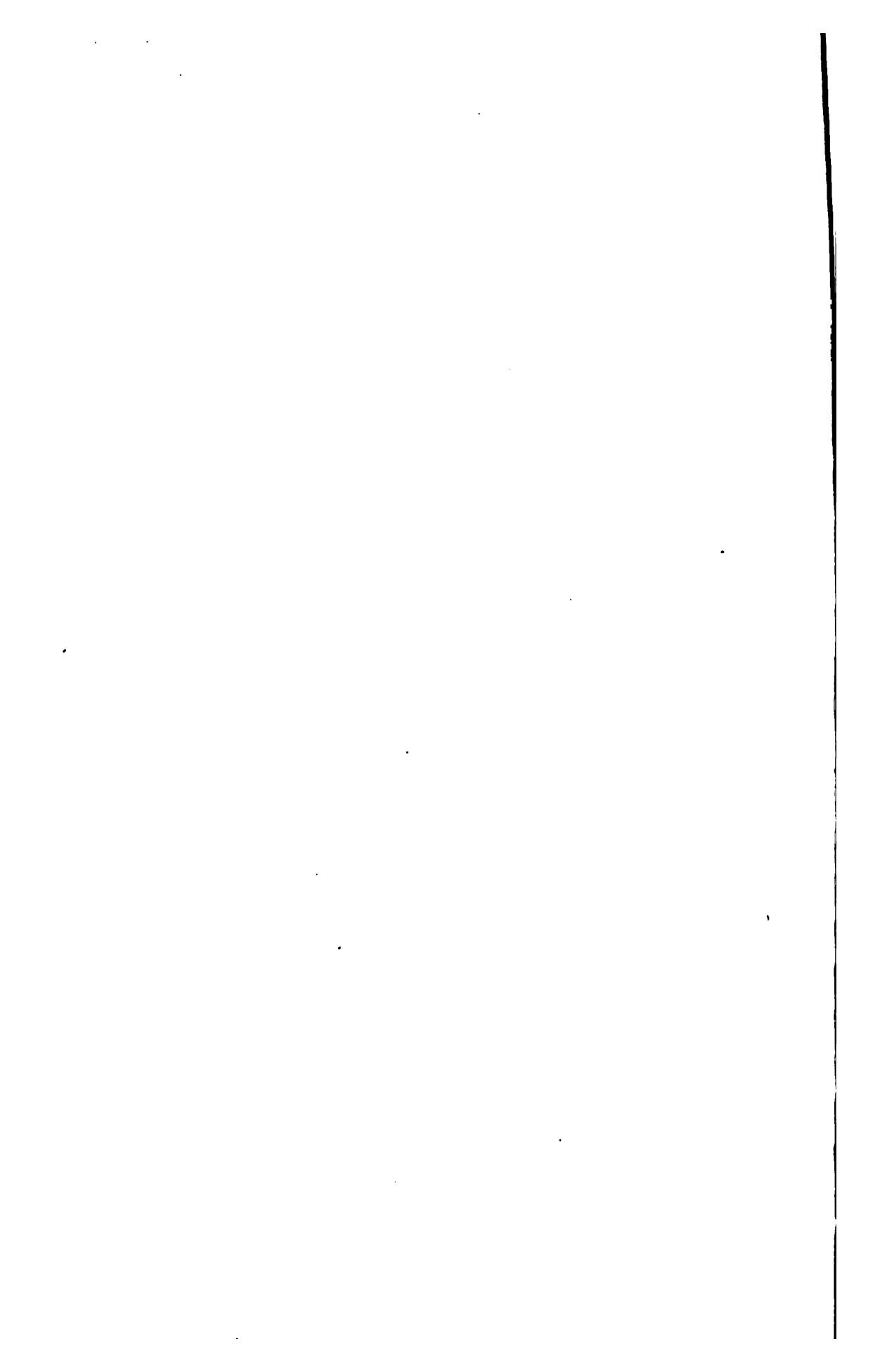
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(Walton: Adult Mammalian Tissue in Simple Plasma.)

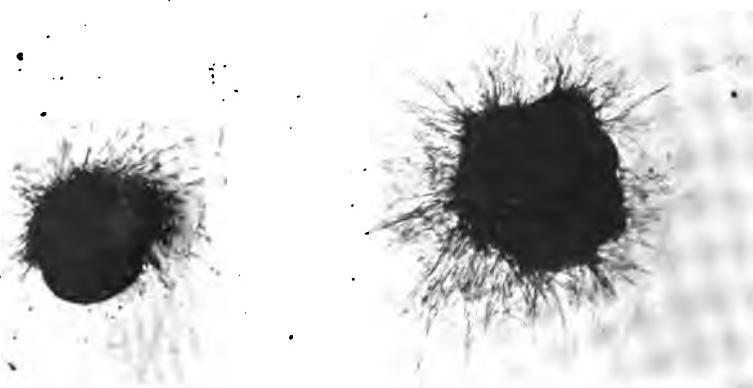
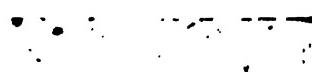




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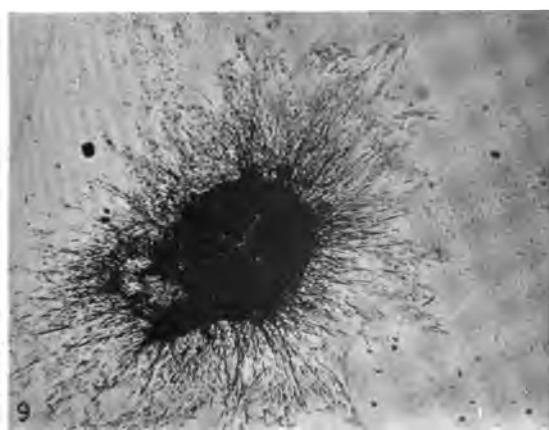


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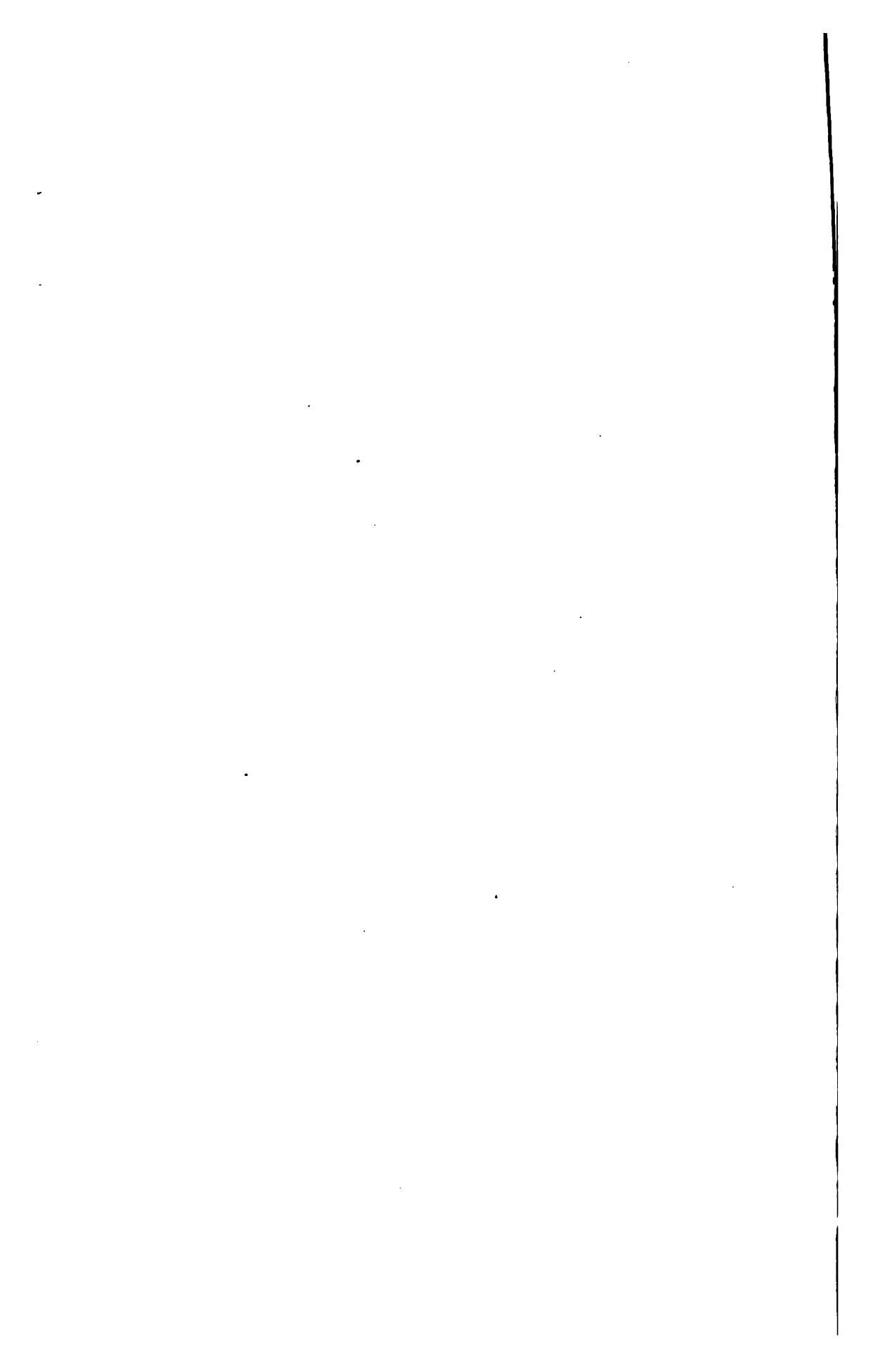


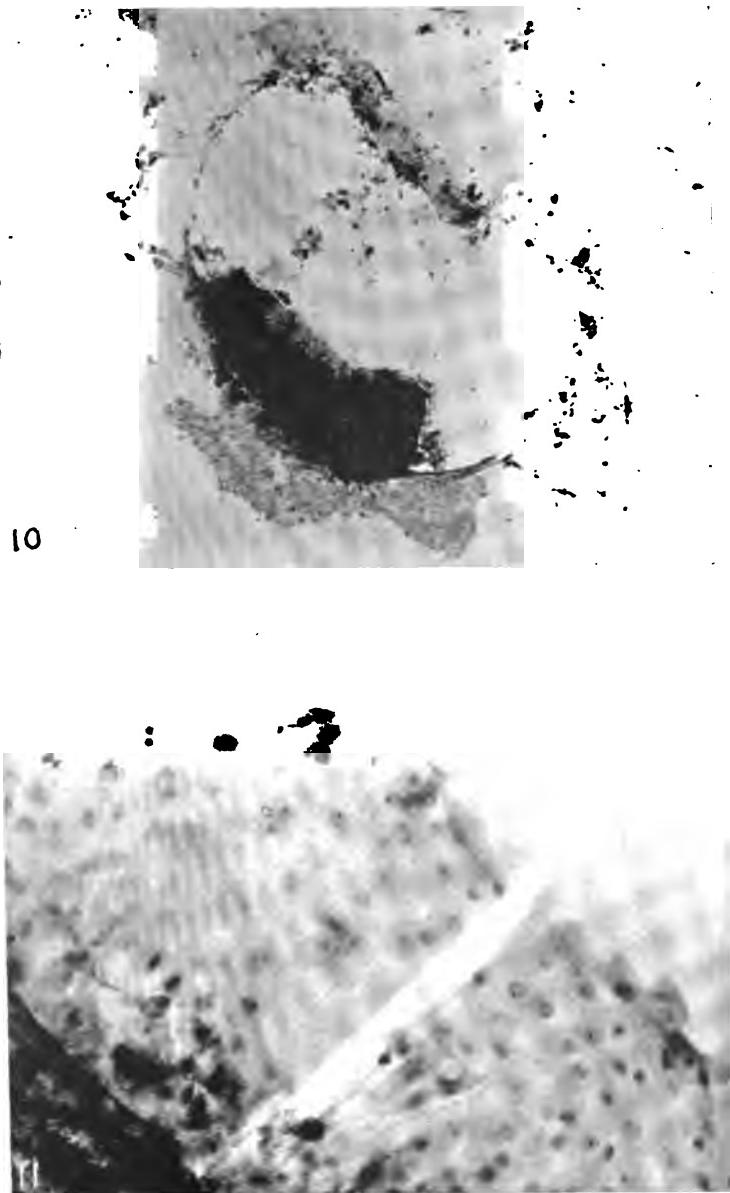
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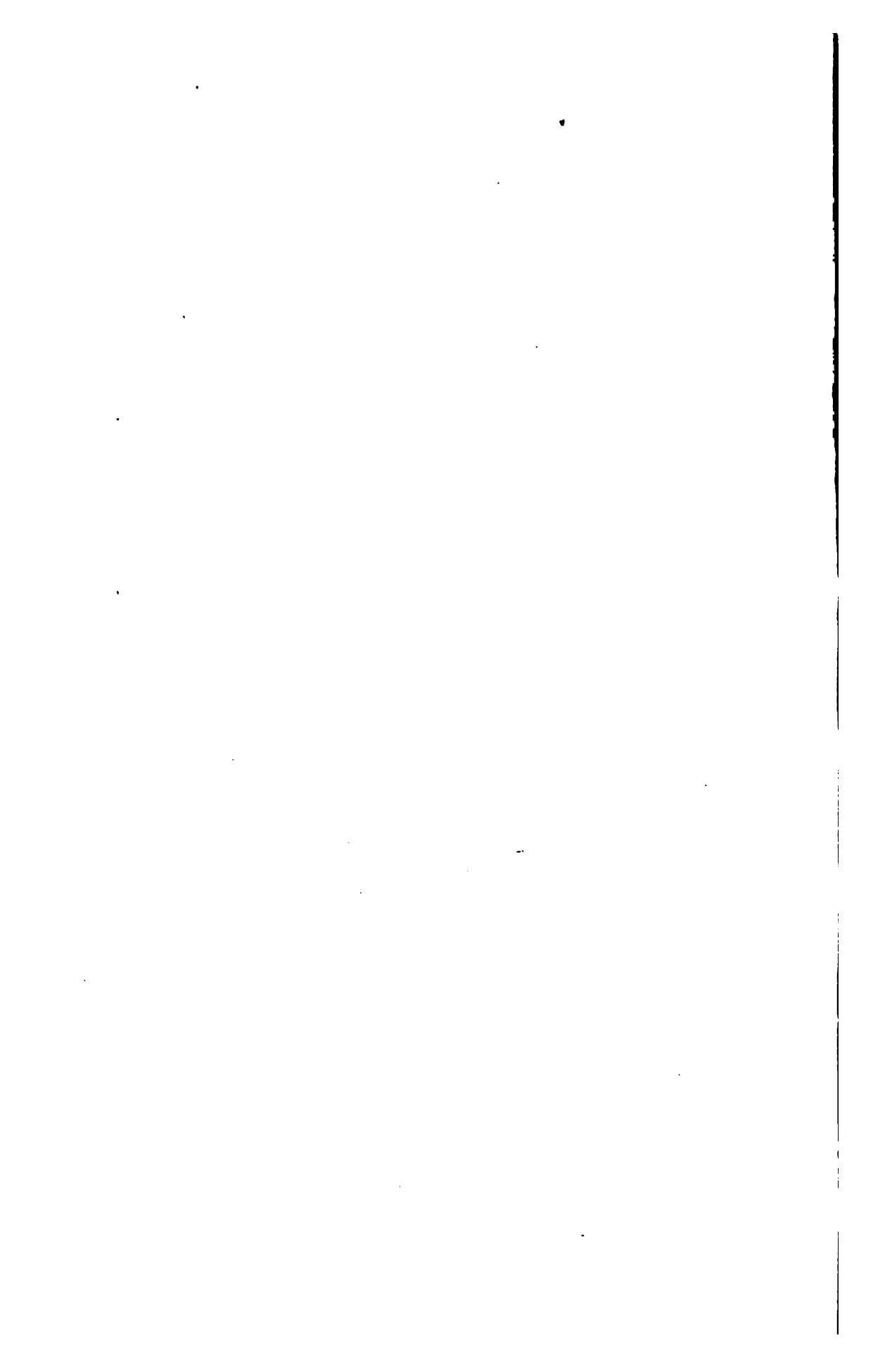


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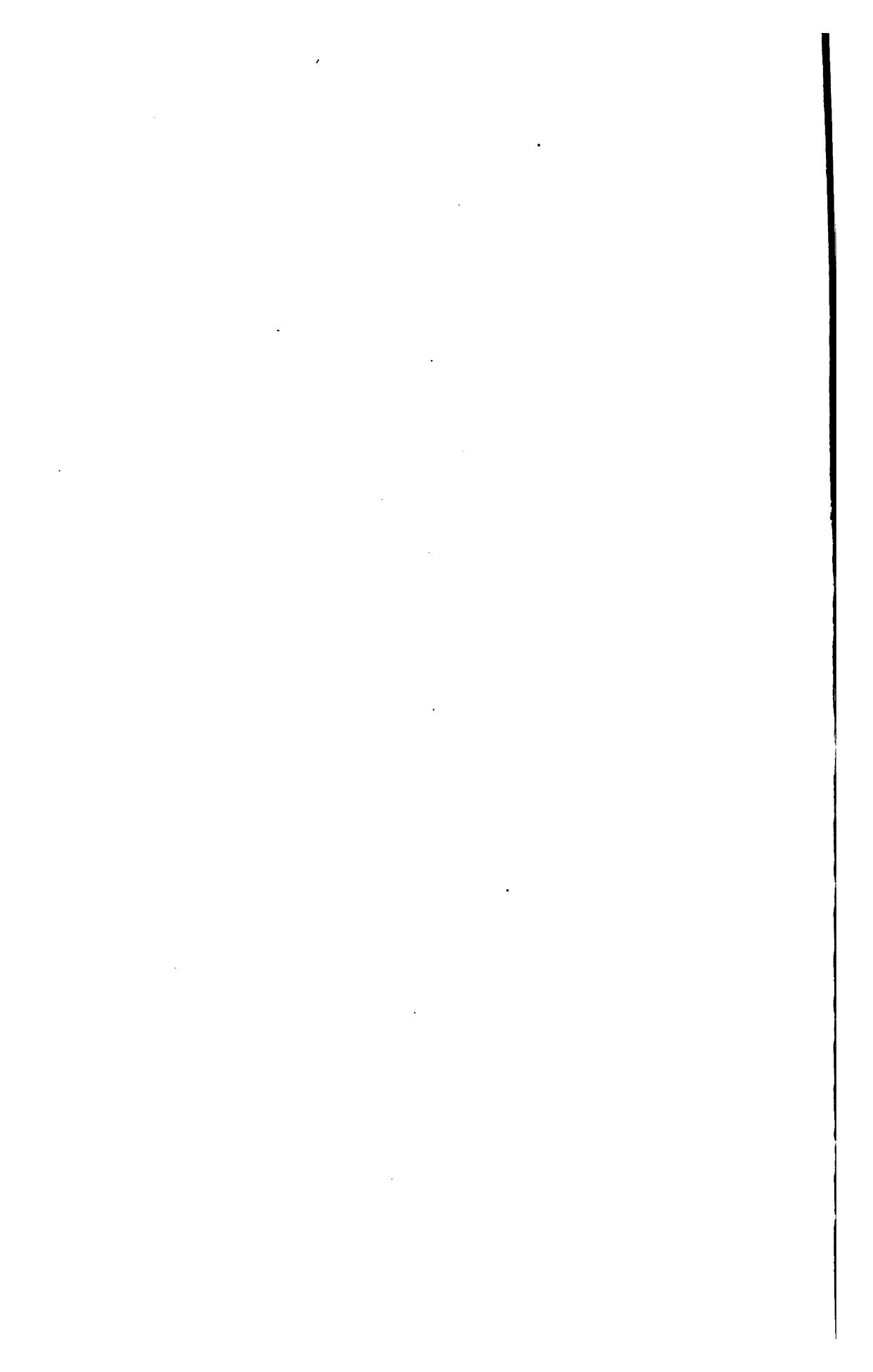


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THE BELLEVUE HOSPITAL.  
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THE BEHAVIOR OF ELASTIC TISSUE IN THE POST-FETAL OCCLUSION AND OBLITERATION OF THE DUCTUS ARTERIOSUS (BOTALLI) IN SUS SCROFA.\*

By J. PARSONS SCHAEFFER, M.D.

(*From the Anatomical Laboratory of the Department of Medicine, Yale University, New Haven.*)

PLATES 27 TO 32.

INTRODUCTION.

Much has been written on the morbid anatomy of blood vessels, including especially such pathological conditions as arteriosclerosis, endarteritis obliterans, thrombo-angiitis obliterans, and arteritis elastica, and reference has occasionally been made to the behavior of elastic tissue in the pathological condition under discussion. Likewise some experimental work has been done in an attempt to simulate diseased conditions in blood vessels, and incidentally some light has been thrown on the nature of the proliferation of elastic tissue in these states. Nevertheless, further work and experimentation are needed in order to elucidate the true conditions involved in the genesis and the proliferation of elastic tissue in postembryonic material.

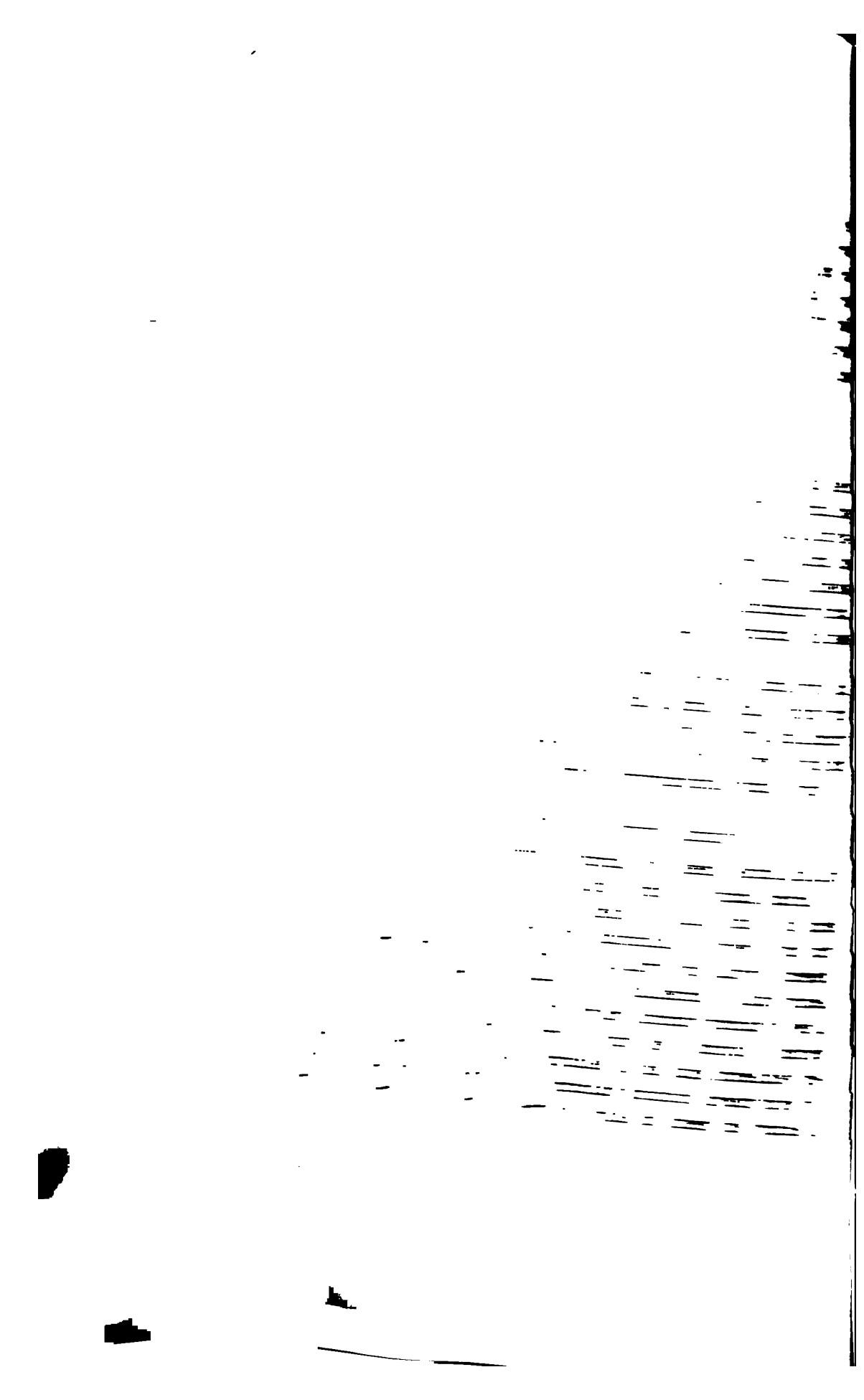
Most of our knowledge concerning the proliferation of elastic tissue in postembryonic material is based on the study of pathological tissues (Langhans, Baumgarten, Thoma, Wendeler, Dmitrieff, Hilbert, Jores, Buerger, and others).

The writer has already reported<sup>1</sup> on the occlusion and ultimate obliteration as such of the ductus arteriosus (Botalli) in man.<sup>2</sup> In

\* Received for publication, October 16, 1913.

<sup>1</sup> Read before the Association of American Anatomists, Cleveland, 1912.

<sup>2</sup> The studies on the occlusion and obliteration of the ductus arteriosus in man were carried on in the Pathological Laboratories of Bellevue Hospital, New York, and in the Anatomical Laboratory of the Yale University Medical School. I wish to express my gratitude to Dr. Charles Norris, Pathologist to Bellevue Hospital, for many courtesies and for the privilege of working in his laboratory and in the Bellevue morgue. I am also indebted to the authorities of the New York Foundling Hospital for the opportunity of working in the hospital morgue.



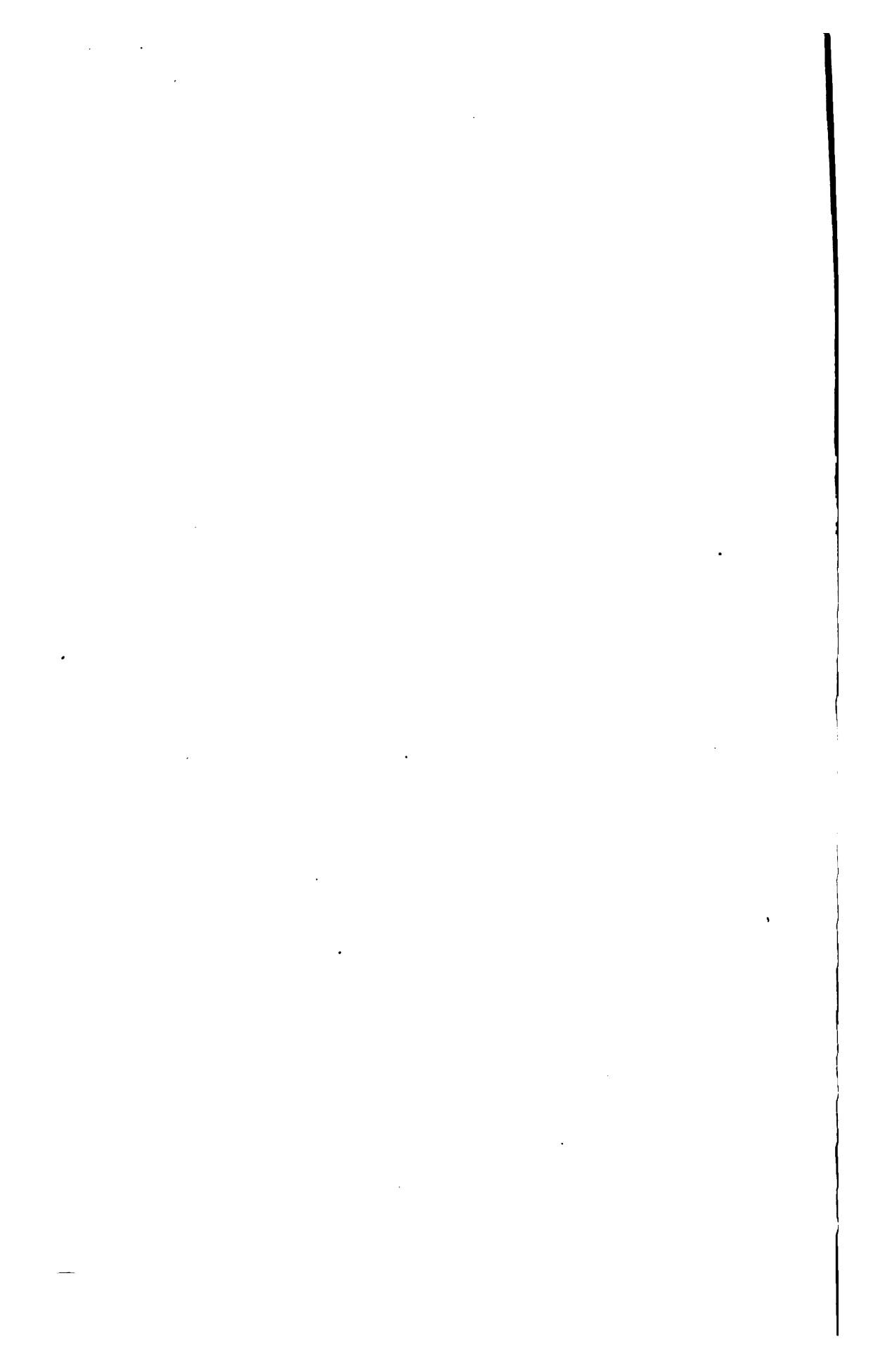
The elastic fibril appears more like a continuous chain than a granule. The arrangement of the elastic fibers in the ductus is more or less longitudinal, and the aortic and pulmonary ends owing to the union of the two ducts. Mall's suggestion that "the granulations of fibrils of elastic tissue" seems to the author to be a better interpretation of the granular appearance.

Study of the early stages indicates that elastic fibers of the ductus arteriosus of *Sus scrofa* are directly differentiated from the connective tissue syncytium. This agrees with the conclusions of Mall and Spalteholz. It is evident that there is a fusion of elastic fibrils in the production of the ligamentum arteriosum.

In the histogenesis of elastic tissue in arteries Mall states that "the exoplasm of the connective tissue syncytium which forms the walls differentiates into two kinds of fibrils, which he calls white fibrous and elastic tissues, respectively. In each cell gives rise to both tissues." I, too, find in the connective tissue syncytium of the lumen of the ductus arteriosus in *Sus scrofa* that in addition to the proliferation of elastic fibers from preexisting connective tissue cells there is also a production of elastic fibrils from non-cellular elements, apparently from connective tissue cells.

The true nature of the process by which white fibrous connective tissue fibers are formed from the exoplasm of connective tissue syncytium is not known. How they come to occupy an intercellular position is yet in dispute. Ferguson, for example, in a recent paper on some experimental work, after dismissing the theory of direct transformation by elongation of mesenchymal cells into connective tissue fibers, and the origin of connective tissue from the ground substance between the cells, apparently adhering to some form of intracellular theory, but he considers that "none of the theories thus far advanced fully meet all of the observed conditions." It is, however, not the purpose of this paper to discuss at length the histogenesis of elastic tissue in embryonic material. Suffice it to say that the material studied gave evidence, as is generally held, that both elastic and white fibrous tissue fibers are laid down in the outlying portion of the protoplasm of the early connective tissue cell.

During the latter half of fetal life the ductus arteriosus of *Sus*



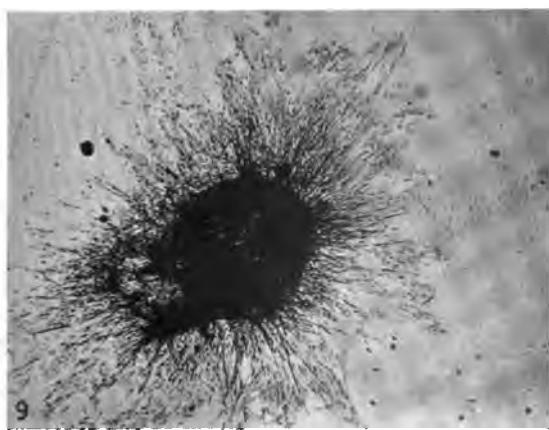
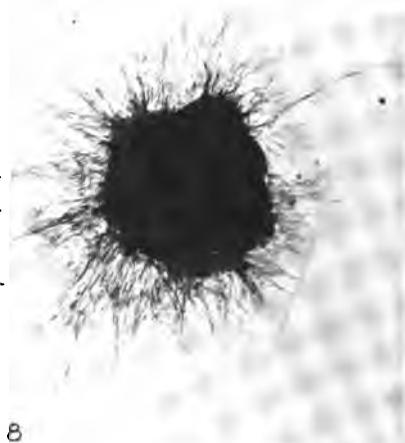
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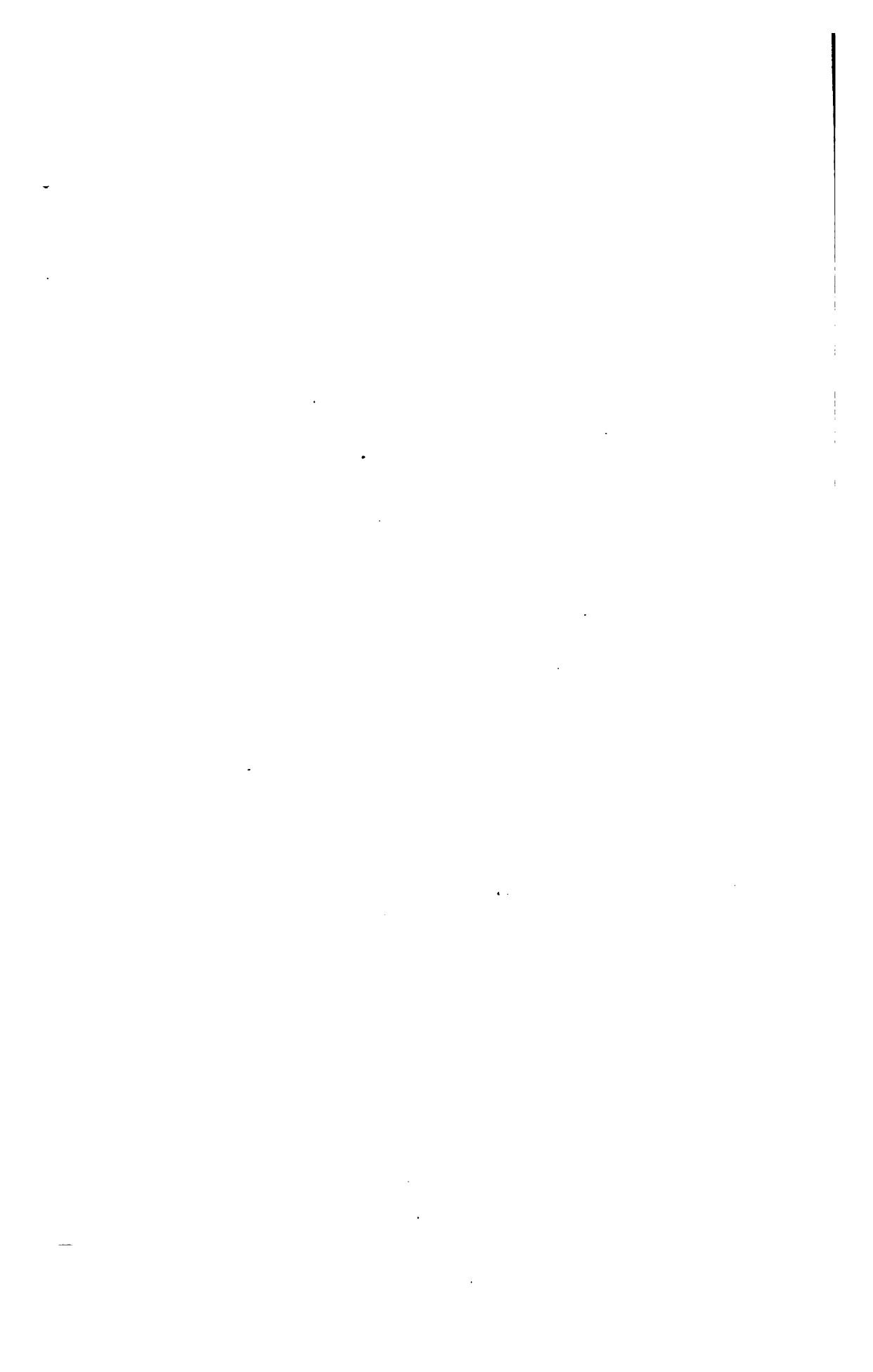
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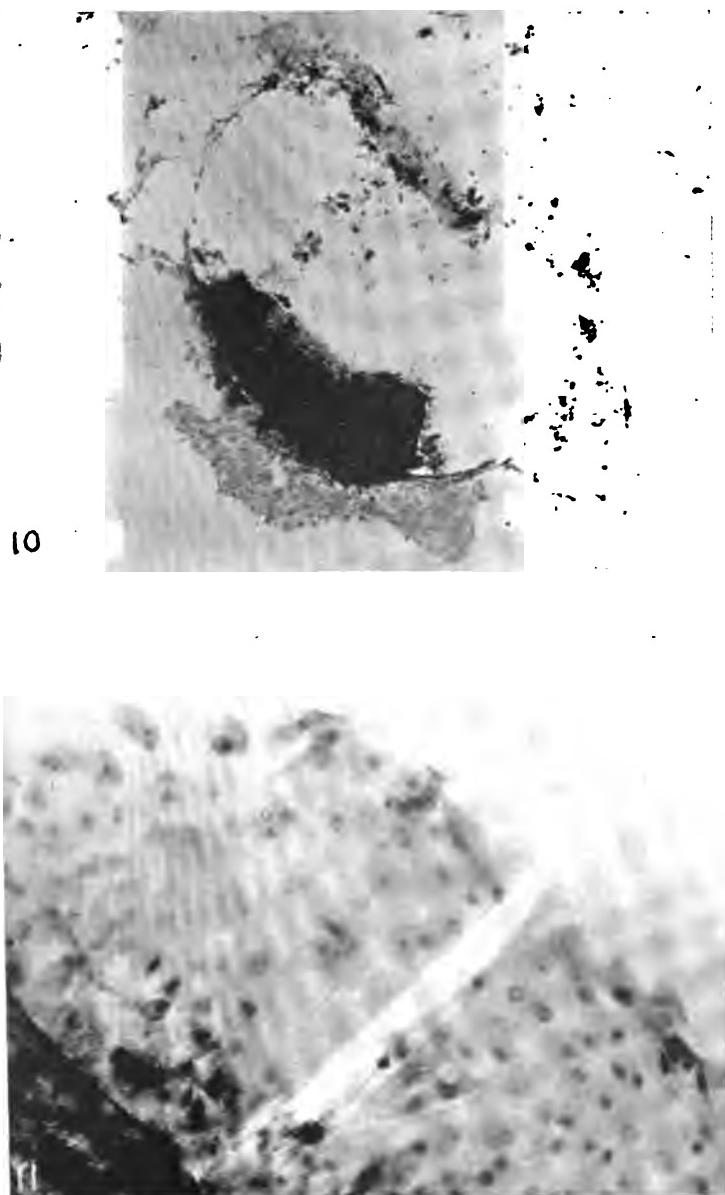


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(Walton: Adult Mammalian Tissue in Simple Plasma.)





(Walton: Adult Mammalian Tissue in Simple Plasma.)

delicate elastic fibers in the slightly thickened intima, the inner elastic membrane was normal and unbroken,—a marked contrast with the condition found in the neonatal ductus arteriosus of *Sus scrofa*. However, as stated previously, these observations are merely preliminary, and it will be necessary to study ligated vessels at repeated intervals up to obliteration and organization of the filled in lumen before any conclusions can be drawn on the behavior of elastic tissue under such conditions.

The preliminary study seems, however, to indicate that in ligations of this character there is early a formation of delicate elastic fibrils in some manner from the cells in the subendothelial layer. Jores believes that the formation of new elastic tissue within the hyperplastic intima in endarteritis is directly due to the activity of the protoplasm of intimal cells.

The conditions that obtain early in the postfetal ductus arteriosus of *Sus scrofa* make the proliferation of elastic tissue more difficult of interpretation. While one does encounter at places very fine elastic fibers amongst the cells of the subendothelial layer, there is also the marked production of elastic lamellæ from the inner membrane previously referred to. It is, therefore, difficult to distinguish between elastic fibers derived from preexisting elastic tissue and those of other origin (figures 7, 8, and 10).

One notices, however, that the finer, irregularly disposed fibrils take the specific stains less deeply than do the lamellæ which are obviously a product of the inner elastic membrane. Furthermore the finer fibrils bear more intimate relations to the protoplasm of the connective tissue cells, *i. e.*, they inmesh the cells. The fine elastic fibers soon become thicker and by migrating become mingled with other fibers. Their real identity then becomes even more vague. Again, there is a similarity between the finer elastic fibers contained in the occluding mass of the lumen of the ductus arteriosus of *Sus scrofa* and those found in the thickened subendothelial stratum of the ligated common carotid arteries previously referred to. It would, therefore, seem that in the occlusion of the ductus there is early a production of elastic fibrils from the protoplasm of the cells contained in the subendothelial layer, as well as

the more extensive production of new elastic fibers by a proliferation of the thickened inner elastic membrane.

As the process of occlusion of the lumen of the ductus advances the new elastic tissue becomes more and more abundant and additional facts are brought out in confirmation of a genesis of elastic fibrils from non-elastic elements. Where the lumen of the ductus is markedly contracted little proliferation of the tissues of the intima is needed, as stated before, to bring about occlusion (figure 13). On the other hand, one encounters ductus and segments of ductus where the contraction is much less, and in order that occlusion may be effected a proliferation of intimal tissues or a thrombosis with subsequent organization, or both, is necessary.

The behavior and disposition of the elastic tissue in the ductus and parts of ductus where a rather large lumen remains to be filled in, is best studied in pigs from the fourth to the ninth week. Then one finds a considerable amount of elastic tissue in the occluding mass, and it is obvious that elastic tissue proliferation plays an important part in the occluding process. A reference to figures 10 and 15 indicates the previously referred to thickening and delamination of the inner elastic membrane. The new elastic laminæ with other proliferated intimal elements have wandered into and occluded the lumen of the vessel. Besides the heavier elastic lamellæ which in part run more or less parallel to the folded old inner elastic membrane, there are present numerous finer elastic fibers, arranged irregularly and somewhat in a network. Often these finer fibers have no connection with the larger and more regular elastic lamellæ and stand independently. Of course, as the process advances there is obviously a fusion of the finer fibers and they become more and more intermingled with and like the coarser fibers. If one is, however, fortunate enough to get the material at opportune stages it is obvious that not all the elastic tissue found in the lumen of the ductus is formed from preexisting elastic tissue.

Again, one finds portions of some ductus in which the occluding process is complicated by the presence of blood amongst the tissues making up the occluding mass, and one questions whether in such portions of the ductus the process is merely a hyperplasia of the intimal tissues. Just what the correct interpretation of the condi-

tion is, is difficult to say. Is there for a time a seeping of blood through the loose occluding mass or is the latter a thrombus undergoing organization? As the occluding mass becomes denser, according to my material, the blood is mostly collected into definite channels which for a time take on the form of definite vessels that run parallel to the original lumen of the ductus. These channels are variable in number and size in different portions of the ductus, and there is no constancy in their disposition, size, or number in different individuals. This is, of course, to be expected owing to the different conditions that usually obtain in different portions of the neonatal ductus. The channels are usually more or less cylindrical, have thin but definite walls, and in many instances a well formed inner elastic membrane, the latter obviously not a product of preformed elastic tissue. Are these channels which contain blood temporary blood vessels formed within a vessel undergoing regression and occlusion, or do they represent canalizations of a thrombus? The fate of these channels and the behavior of the elastic tissue adds some light on these questions.

The vessels or channels frequently found in the occluding mass are for the most part short lived (figures 11 and 12). As the occluding mass of the lumen of the ductus becomes denser there is also apparently a proliferation of the tissues composing the walls of the channels. Ultimately these processes cause the channels to suffer the same fate as the mother ductus, *i. e.*, occlusion and obliteration. In place of the channels nests of cells are subsequently found (figure 12), and when specific stains are used definite masses of elastic tissue are found here and there in the mass occluding the lumen, obviously the site of the former blood channels (figure 17).

On the whole, the channels do not behave like canalizations of thrombi observed in pathological material. Whether the ultimate fate of canalized channels in thrombi is, in some instances, similar to the blood channels frequently found in the mass occluding the lumen of the ductus arteriosus of *Sus scrofa* I have been unable to observe. Hematoxylin and eosin-stained sections of such segments of ductus, as above referred to, look somewhat like organizing thrombi (figure 11). In the latter there is some round cell infiltration and a considerable amount of blood diffused through the mass.

In later specimens the blood is found for the most part in definite channels which are found in the mass. Fibroblastic cells, which probably took origin from the proliferated endothelial lining or from the cells in the hyperplastic subendothelial stratum, are also present in the mass. Elastic tissue is present in abundance. Here and there, especially around the blood channels in the mass, are found elements that look and behave like unstriped muscle cells. Modified endothelial cells are also encountered. However, only by staining sections from the same segment of the ductus with specific stains is the richness of elastic tissue in the occluding mass and the marked delamination of the thickened internal elastic membrane brought out. The disposition of the elastic tissue was previously referred to (figures 11 and 15).

Buerger, who recently made a study of blood vessels from cases of presenile gangrene, considers the obliterating process found in such cases a thrombo-angiitis obliterans. He believes that the behavior of the elastic tissue distinguishes an obliterating arteriosclerotic process (arteritis obliterans) from a thrombo-angiitis obliterans. In the former there is, of course, among other things a marked hyperplasia of the intimal tissues; in the latter primarily an organization of a thrombus. Buerger finds in his cases of thrombo-angiitis obliterans, "either a total absence of elastic fibers, or when such are present, a growth of fibers around the larger canalizing vessels, particularly about those which are thick walled and old." I recently had the opportunity of examining a few small vessels taken from a case of presenile gangrene and found in the sections studied that the organizing thrombi contained no elastic tissue. The inner elastic membrane was sharply demarcated and not delaminated, as in the neonatal ductus of the pig. However, the material was too limited to permit one to draw conclusions in this connection. Jores in some experimental work apparently demonstrated elastic fibers in organizing thrombi. As stated previously, the work on the behavior of elastic tissue in ligations, organization of thrombi, etc., is as yet incomplete, as is also the work on the postfetal occlusion of blood vessels. Experimental studies bearing further on these problems are now in progress, the results of which will be reported subsequently.

## ADULT DUCTUS ARTERIOSUS.

In the adult the ductus arteriosus of *Sus scrofa* not merely presents an occluded lumen, but the identity of the vessel as such is lost. However, one can often detect the site of the former lumen. The elastic tissue throughout the ductus instead of appearing in distinct fibers and lamellæ is broken up into fragments and may appear in small masses, the latter more especially at the site of the former lumen (figure 14). The whole ductus has undergone regression and is now essentially a fibro-elastic cord connecting up the pulmonary and systemic aortæ. Here and there unstriped muscle fibers remain. Small blood vessels supply the tissues of the retrogressed ductus.

## SUMMARY.

A study of the histogenesis of elastic tissue in the embryonic ductus arteriosus of *Sus scrofa* is in accord with the theory that elastic fibrils are directly differentiated in the outlying portion of the protoplasm of the early connective tissue cell.

In the occlusion of the postfetal ductus arteriosus of *Sus scrofa* there is early a hypertrophy of the internal elastic membrane. Subsequently there takes place a marked delamination of the thickened internal elastic membrane in the production of new and independent elastic fibers and lamellæ. The formation of new elastic fibers from preformed elastic tissue is most abundant where the postfetal contraction of the ductus arteriosus is least marked. These new elastic fibers play an important part in the occlusion of the lumen of the postfetal ductus.

Aside from the extensive formation of elastic fibers from preformed elastic tissue, in the occlusion of the lumen of the postfetal ductus arteriosus of *Sus scrofa*, there are also some elastic fibrils formed from non-elastic elements, apparently from connective tissue cells.

In some recent preliminary work on ligations of the common carotid artery there was found, after an interval of from eight to twelve days, at some points between the ligatures, a slight but obvious cellular thickening of the so-called subendothelial stratum. Some of these connective tissue cells may have wandered from the

other coats of the vessel, through the inner elastic membrane into the subendothelial stratum; others may have proliferated from cells *in situ*. Specific stains revealed near the periphery of some of these cells, *i. e.*, in the outlying portion of the exoplasm, very delicate elastic fibrils, apparently the product of protoplasmic activity.

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## EXPLANATION OF PLATES.

The first fourteen figures are photomicrographs of cross-sections of the ductus arteriosus. The sections illustrate the various stages in the occlusion of the lumen of the ductus and its obliteration as such. Figures 1 to 10 inclusive and 13 and 14 are from sections stained with either Weigert's elastic stain and counterstained with picrofuchsin, or some form of orcein stain. Figures 11 and 12 are from sections stained with hematoxylin and eosin. Figures 15, 16, and 17 are camera lucida drawings from Weigert-picrofuchsin stained sections.

## PLATE 27.

Figs. 1 to 3. Cross-sections of human ductus arteriosus from infants aged 30, 21, and 102 days, respectively. Note in figures 1 and 2 the pad-like thickening of the intima and the abundance of elastic tissue in this thickening. In figure 3 the inner elastic membrane is thickened at places, with denser laminae bounding ectally and entally a finer network of elastic fibrils. The subendothelial stratum is also hyperplastic.

FIG. 4. Cross-section of a fetal ductus arteriosus from a pig 24 cm. long. Note the multilaminar condition of the inner elastic membrane on one side.

Figs. 5 to 8. Cross-sections taken from various levels of the ductus arteriosus of a postfetal pig fourteen days old. In most of the sections the early, characteristic thickening and delamination of the inner elastic membrane is well shown. The multilaminar condition is well advanced in sections (figures 7 and 8). The latter are from the aortic thirds of the ductus. The pulmonary end of the ductus was even farther advanced in the production of new lamellæ from the inner elastic membrane (compare figure 9). In figure 5 a mass of blood is found in the lumen of the ductus, and from all appearances blood was flowing through the lumen of the vessel when the specimen was secured. The characteristic contraction so often seen in the pig ductus after birth is illustrated in figure 6.

Note especially the fine network of elastic fibers in the subendothelial stratum in the section represented in figure 8. The finer elastic fibrils are readily distinguished from the heavier lamellæ which are a product of the preformed inner elastic membrane.

## PLATE 28.

FIG. 9. Cross-section from the pulmonary thirds of the ductus arteriosus from a pig fifteen days old. The lumen is more or less irregular, due to contraction, but a considerable amount of intimal hyperplasia would be necessary to occlude the lumen. The inner elastic membrane has already thickened and at places split into a number of new laminae.

FIG. 10. Cross-section of the ductus arteriosus from a pig eight weeks old. The lumen of the ductus is entirely filled in with a mass of new tissue. Note the irregularity of the former lumen, and the new, heavy elastic laminae formed from the old inner membrane. Here and there blood channels with definite walls and in some instances with well formed inner elastic membranes are found in the occluding mass. Note also the finer and less deeply stained network of elastic fibrils throughout the occluding mass.

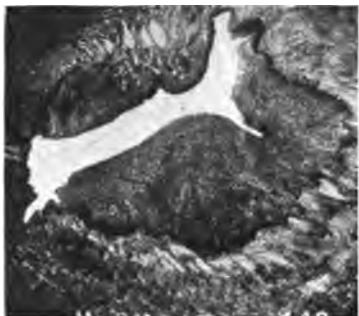


FIG. 1.



FIG. 5.



FIG. 2.



FIG. 6.



FIG. 3.



FIG. 7.

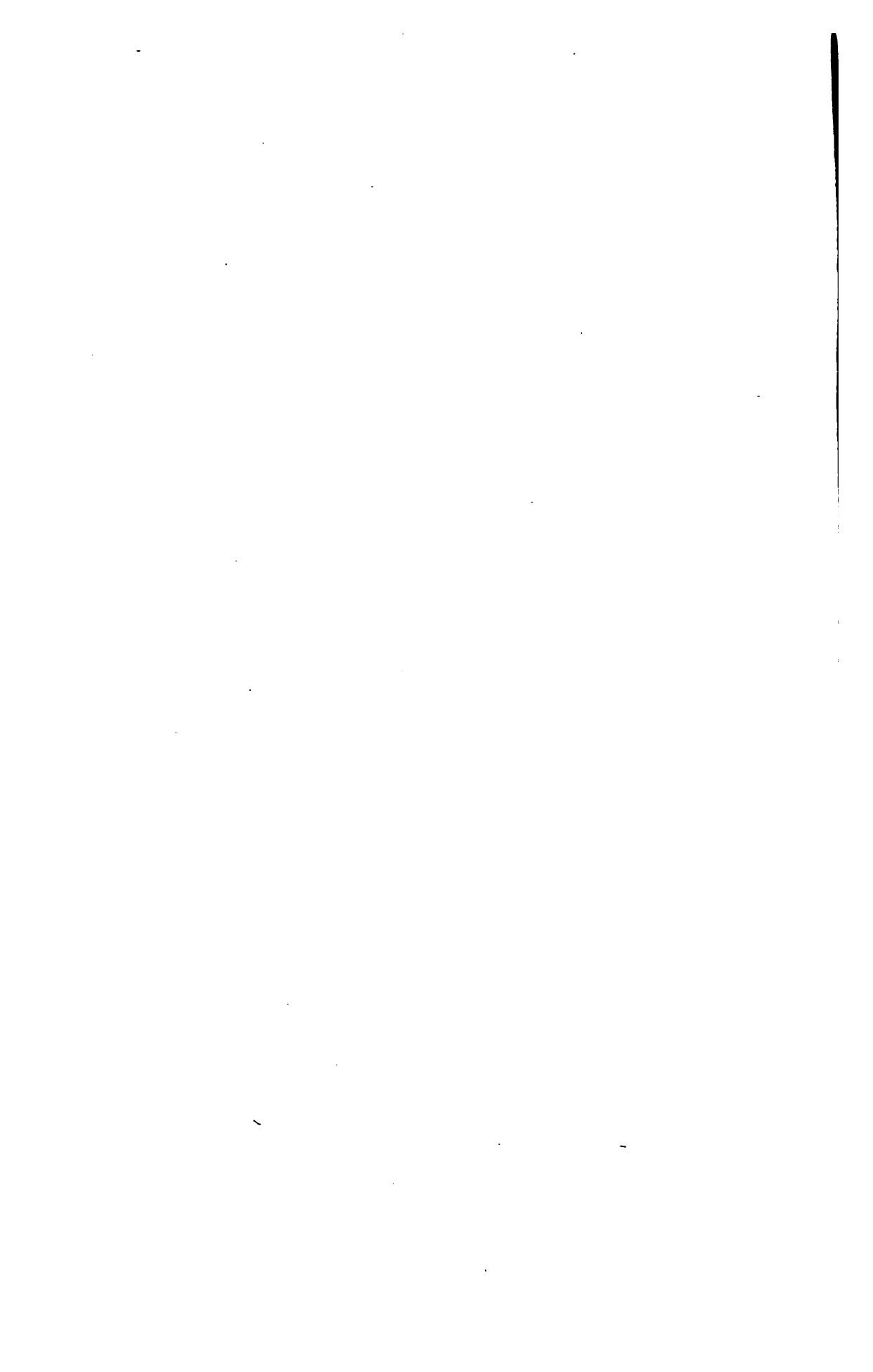


FIG. 4.



FIG. 8.

(Schaeffer: Behavior of Elastic Tissue in Postfetal Occlusion.)



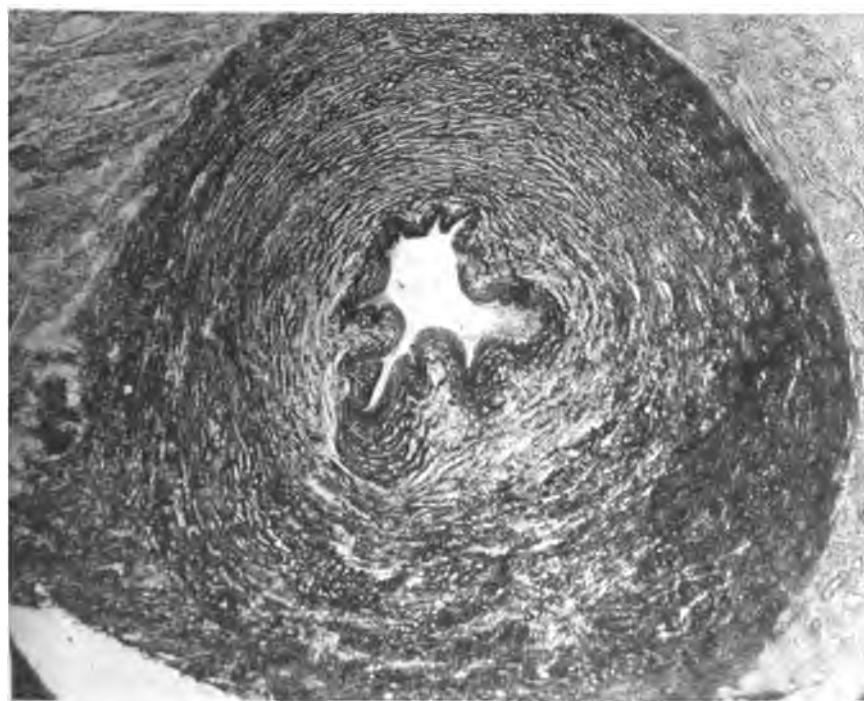


FIG. 9.

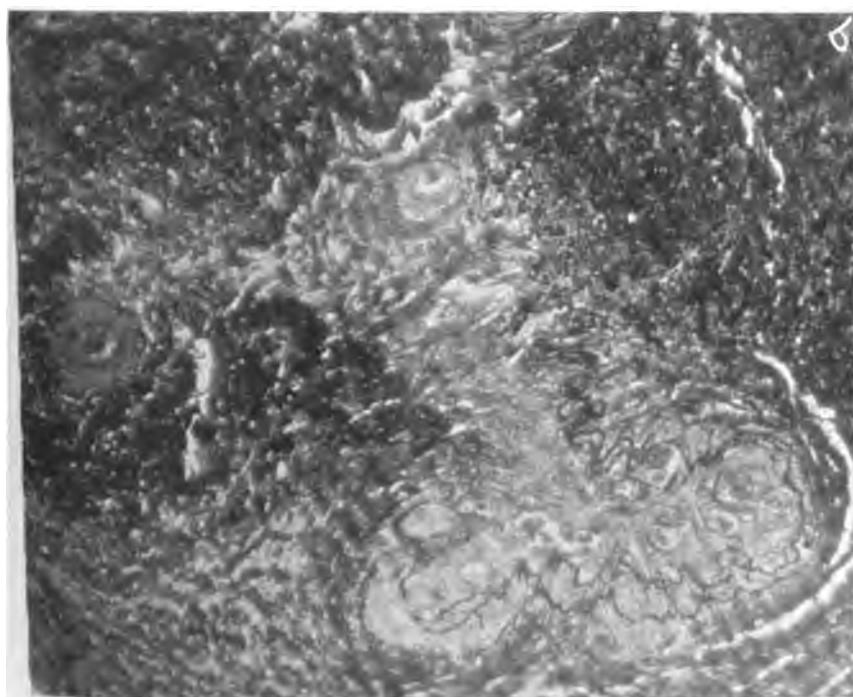


FIG. 10.

(Schaeffer: Behavior of Elastic Tissue in Postfetal Occlusion.)

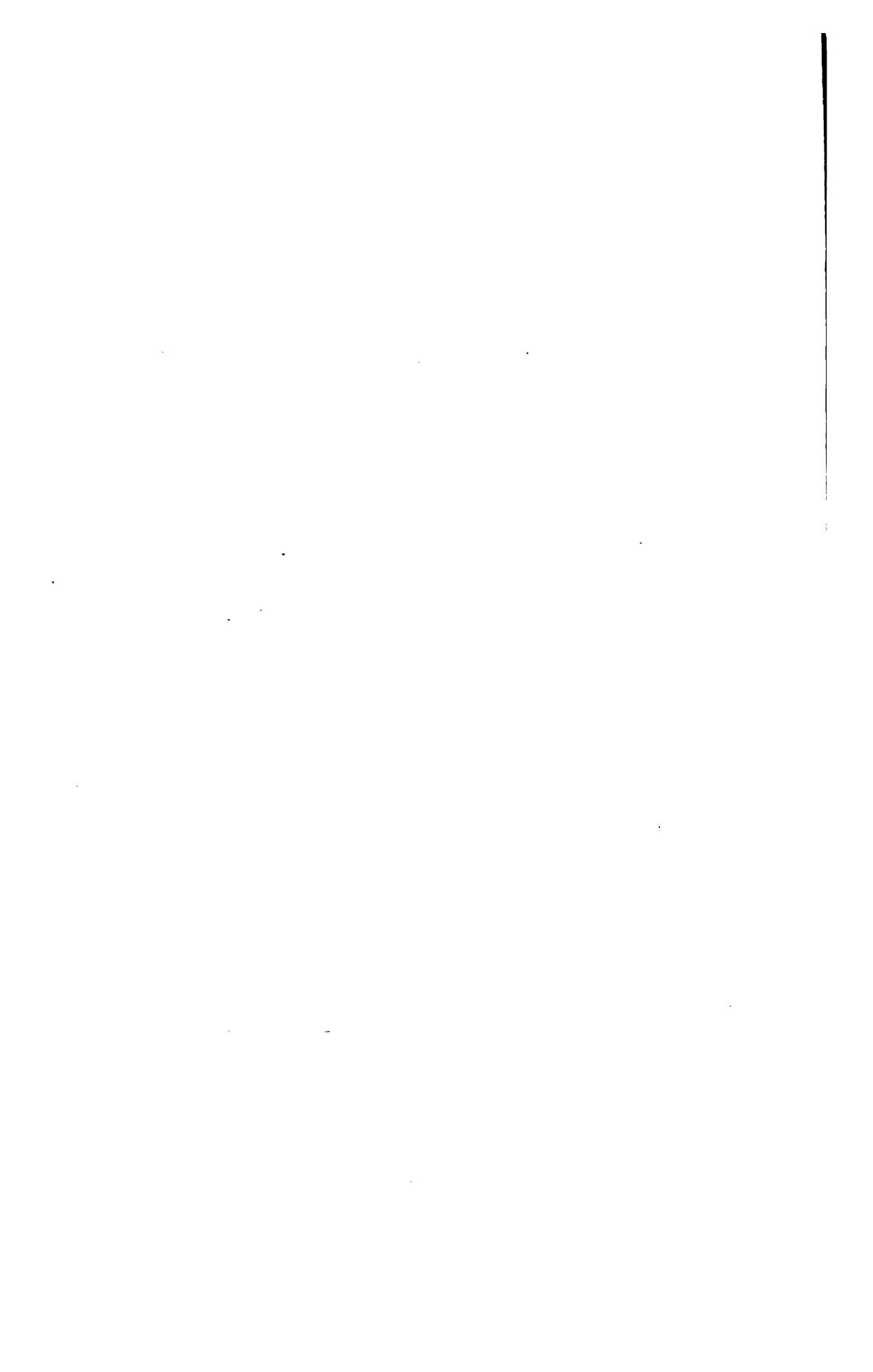




FIG. 11.

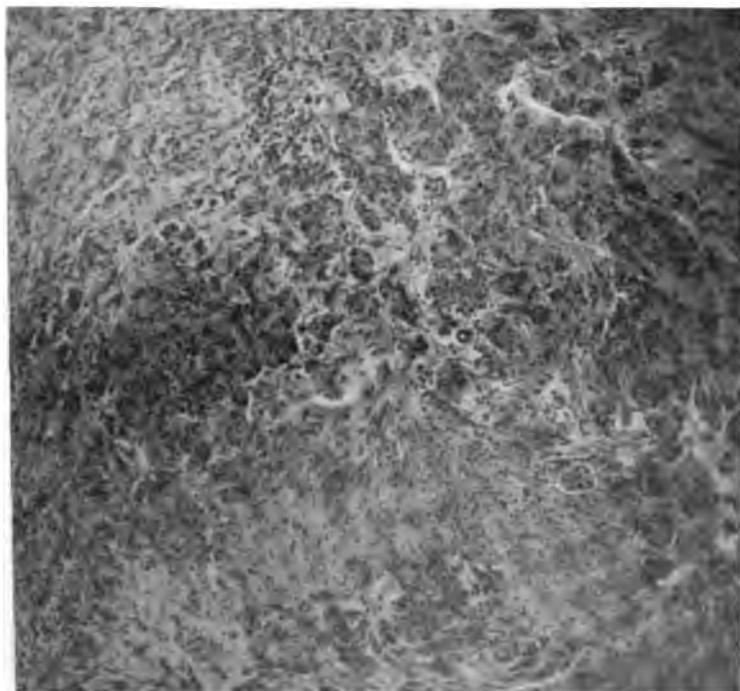
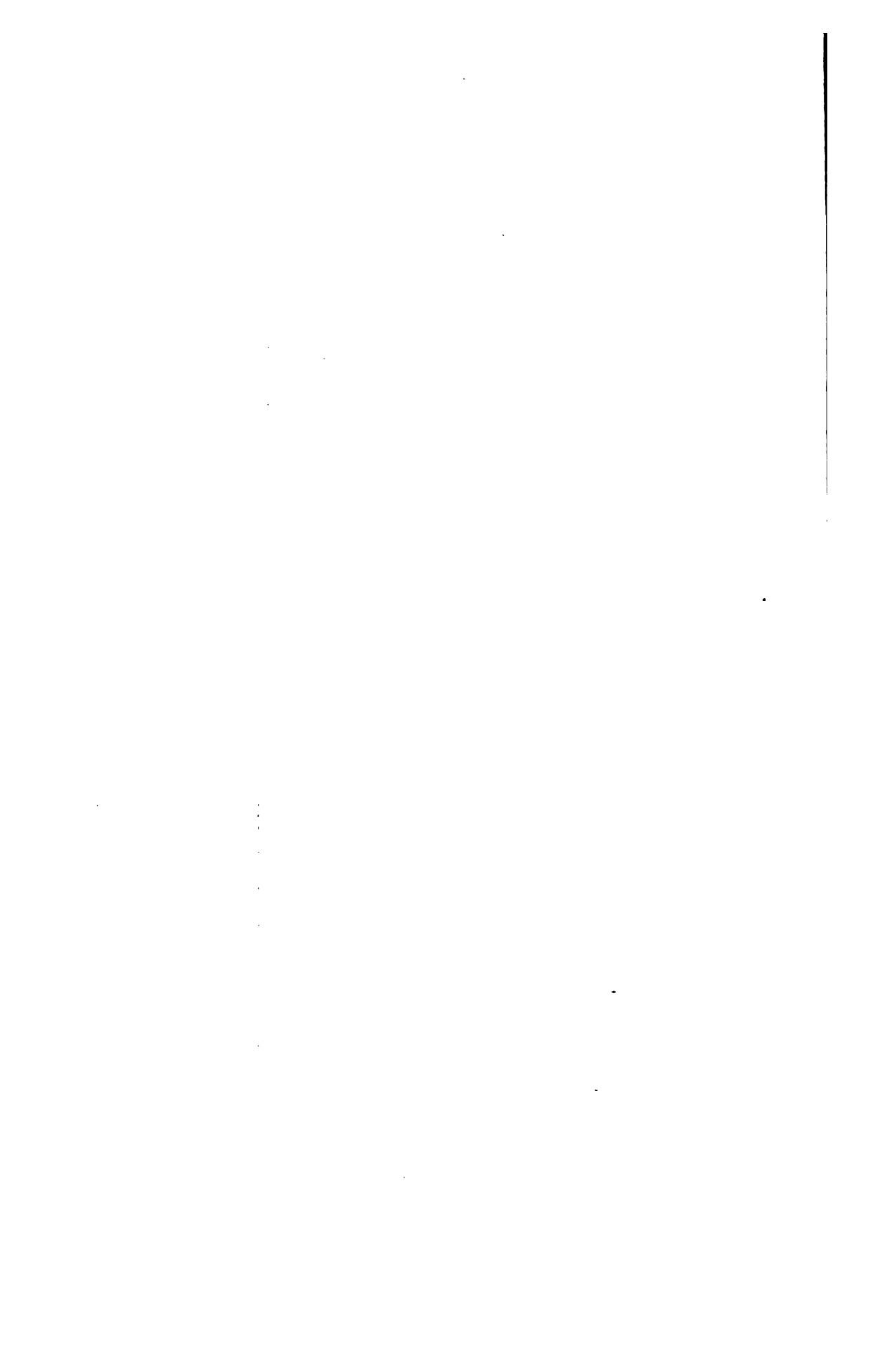


FIG. 12.

(Schaeffer: Behavior of Elastic Tissue in Postfetal Occlusion.)



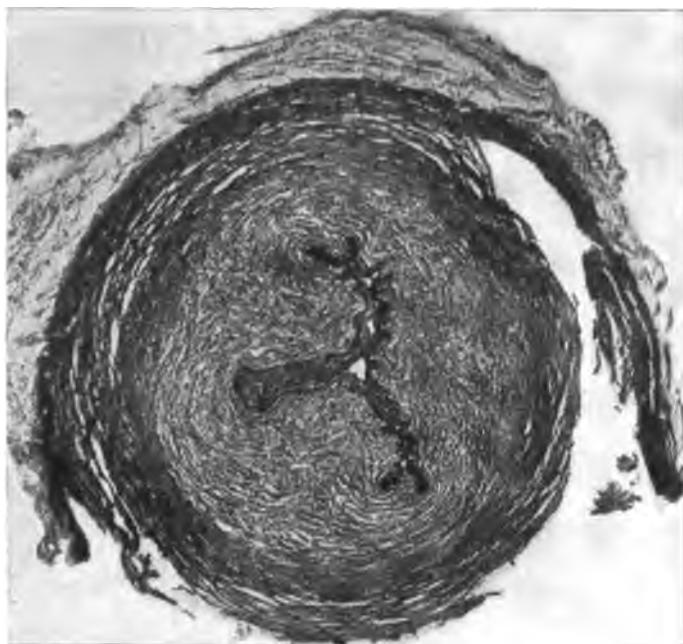


FIG. 13.

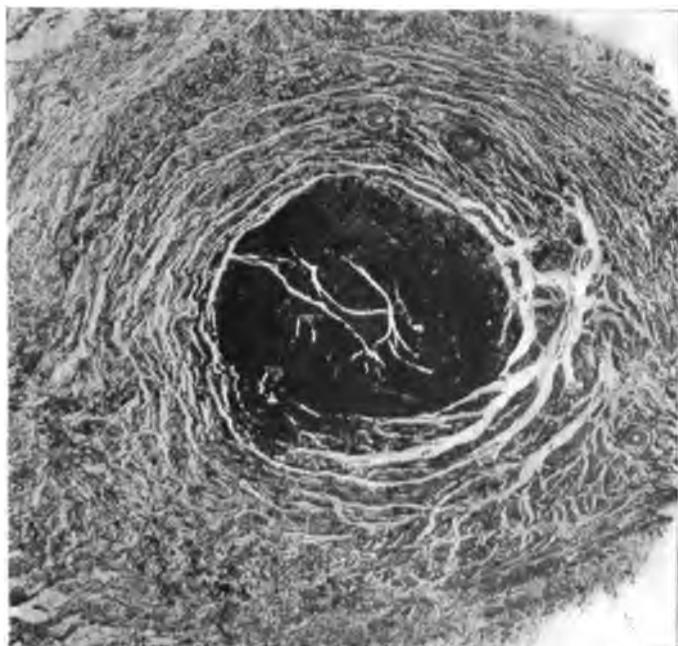
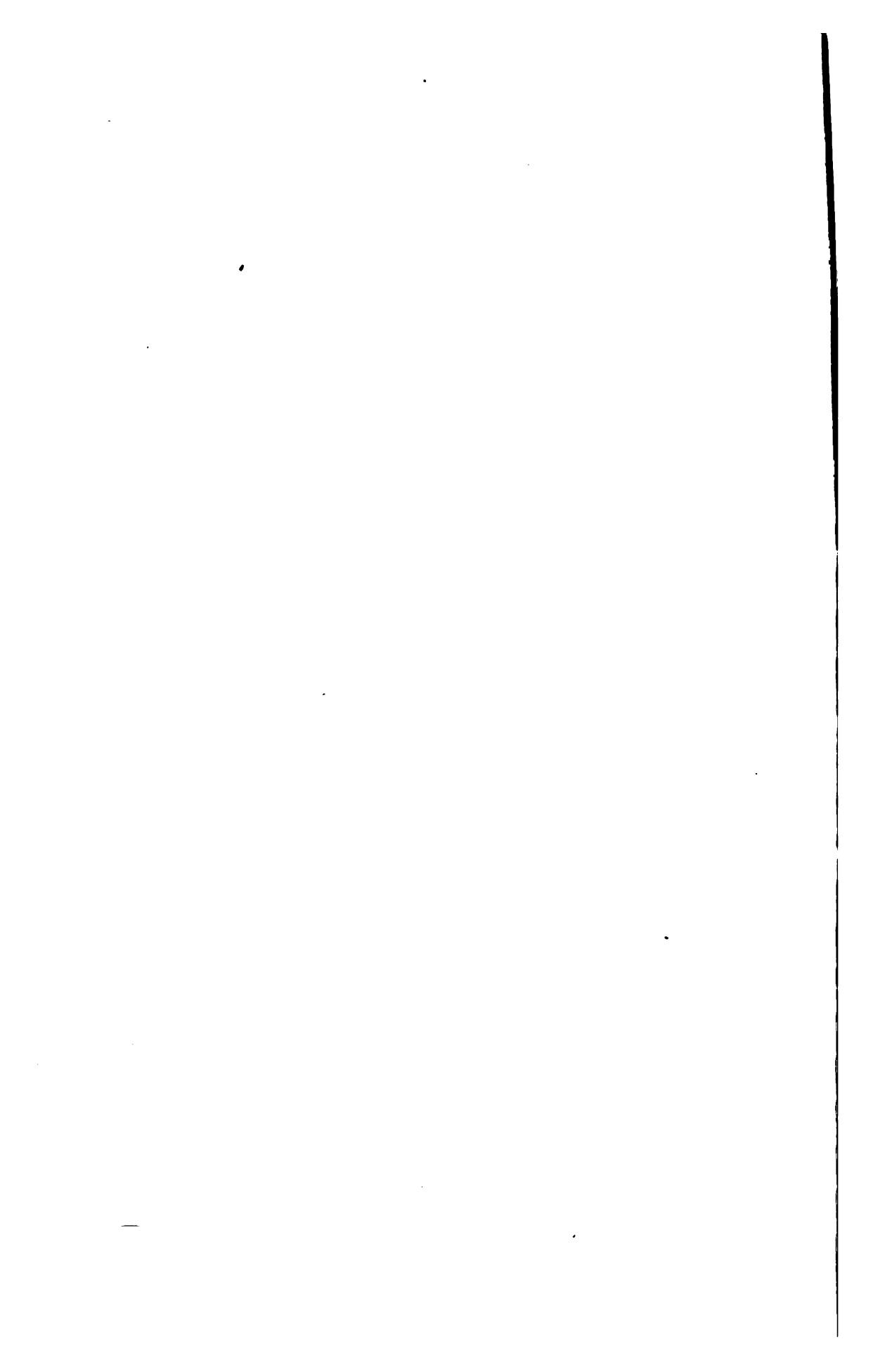


FIG. 14.

(Schaeffer: Behavior of Elastic Tissue in Postfetal Occlusion.)



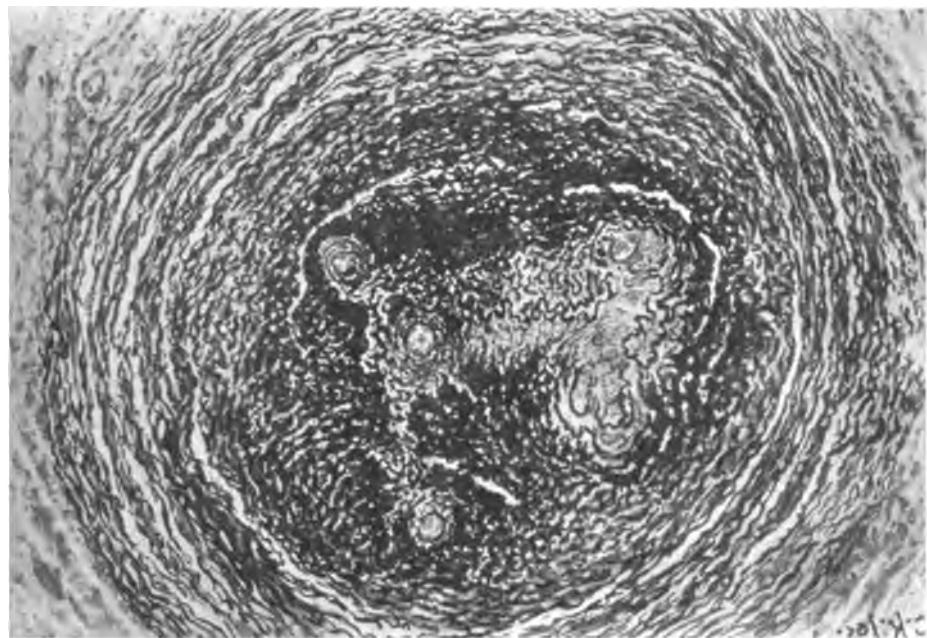


FIG. 15.

(Schaeffer: Behavior of Elastic Tissue in Postfetal Occlusion.)



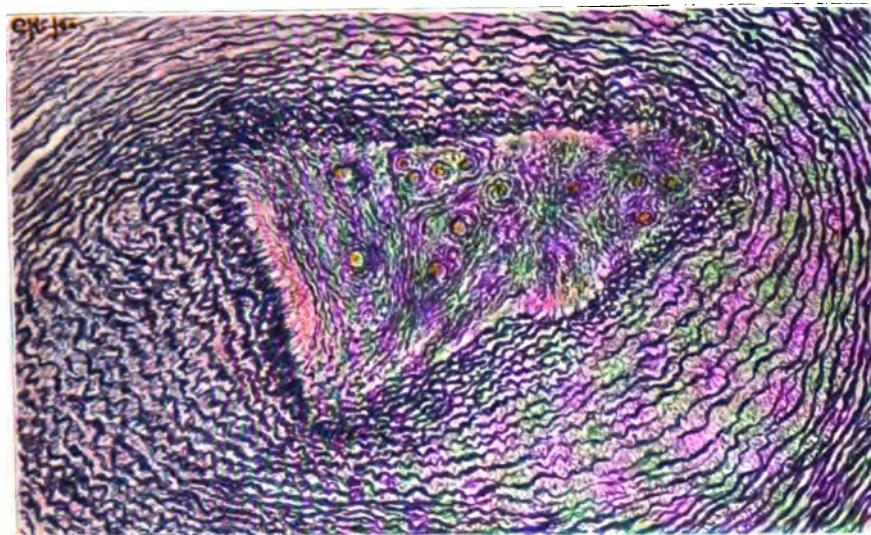


FIG. 16.

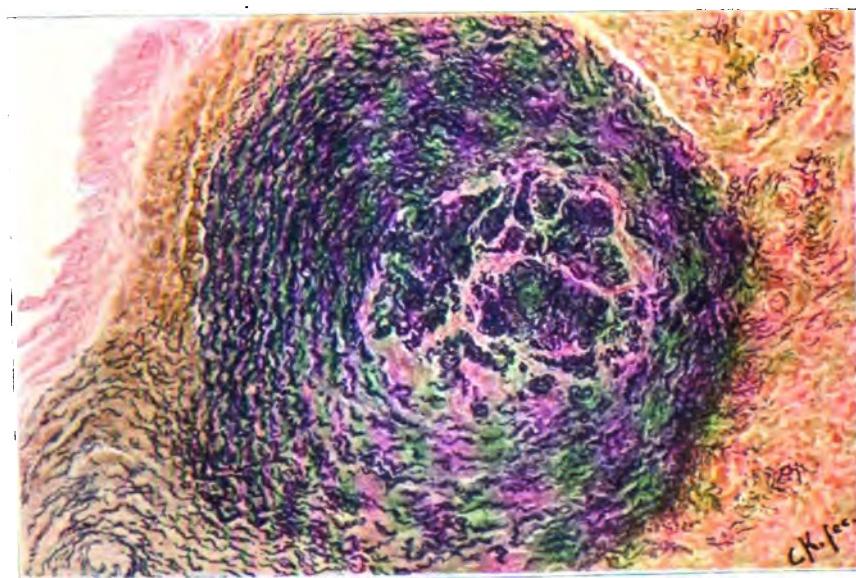


FIG. 17.

(Schaeffer: Behavior of Elastic Tissue in Postfetal Occlusion.)

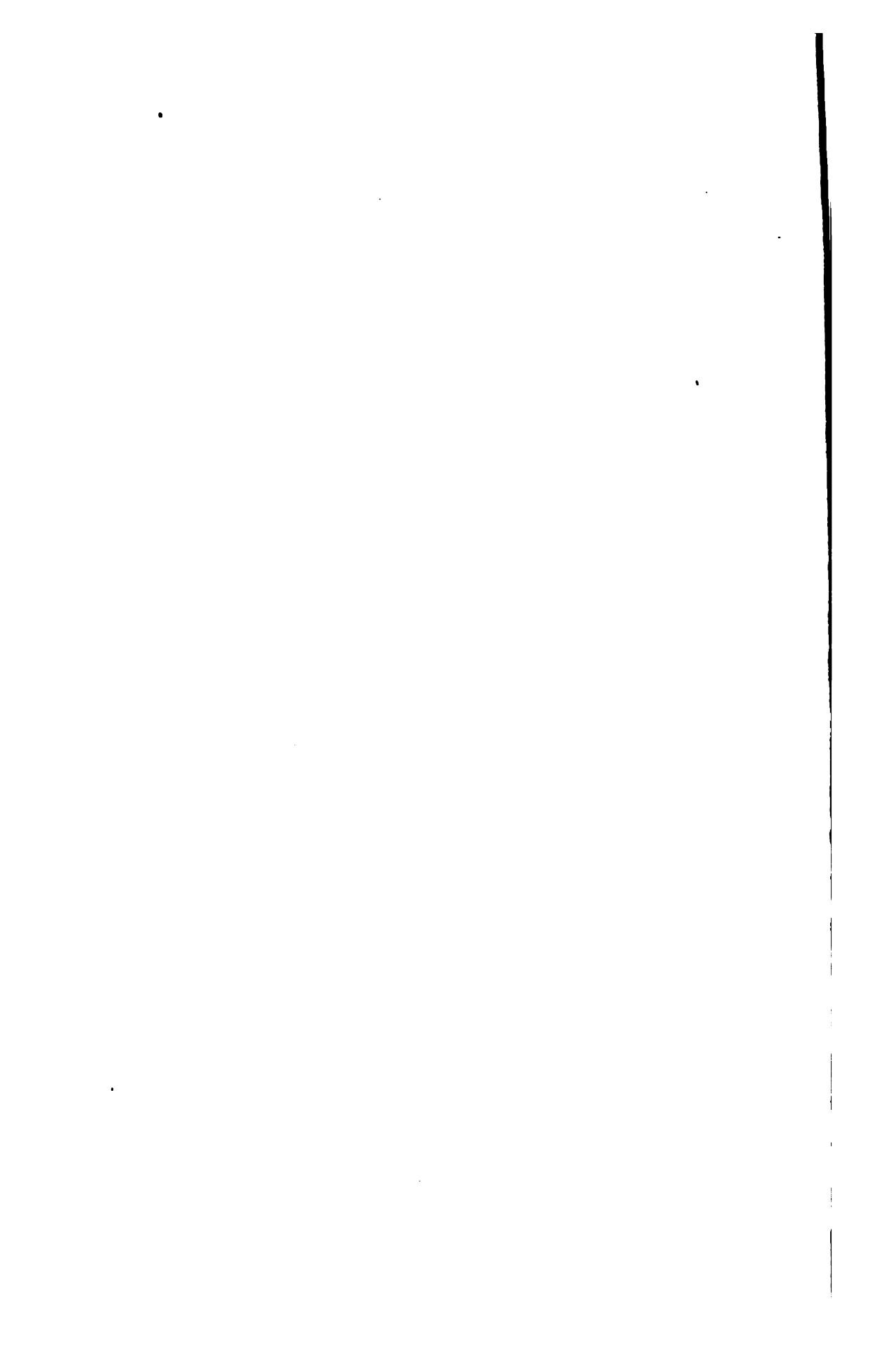


PLATE 29.

FIG. 11. Cross-section of the ductus arteriosus from a pig eight weeks old. The section is stained with hematoxylin and eosin. Note especially the complete occlusion of the lumen of the vessel and the large number of blood channels in the occluding mass. The mass looks somewhat like an organized thrombus with subsequent canalization (compare figure 10). Both sections are from the same segment of the same ductus.

FIG. 12. Cross-section of the ductus arteriosus from a pig ten weeks old. The section is stained with hematoxylin and eosin. The lumen of the ductus is entirely occluded and obliterated. Note especially that in place of blood channels, illustrated in figures 10, 11, and 15, there are nests of cells, obviously the site of earlier vascular channels. Smaller vessels have now grown into the new tissue to supply it.

PLATE 30.

FIG. 13. Cross-section of the ductus arteriosus of a pig two days old. The section is stained for elastic tissue. Note the marked contraction of the lumen in this instance. The inner elastic membrane is already somewhat thickened and at places split into layers.

FIG. 14. Cross-section, stained for elastic tissue, of the ductus arteriosus from an adult pig. Note the great amount of broken up elastic tissue in the occluding mass in comparison with the amount remaining elsewhere in the retrogressed ductus.

PLATE 31.

FIG. 15. Camera lucida drawing of a Weigert-picrofuchsin stained section of the ductus arteriosus from a pig sixty days old. The delamination of the thickened inner elastic membrane is here well illustrated. Aside from the heavier elastic lamellæ in the occluding mass there are many finer elastic fibrils present, less deeply stained.

PLATE 32.

FIG. 16. Camera lucida drawing of a cross-section from the aortic thirds of the ductus arteriosus from a pig sixty-three days old. The section was stained with Weigert's elastic tissue stain and counterstained with picrofuchsin. Note the abundance and the disposition of the elastic tissue and the definite vascular channels in the occluding mass.

FIG. 17. Camera lucida drawing of a cross-section from the midportion of the ductus arteriosus from a pig eleven weeks old. Note the disposition of the elastic tissue at this time (compare figure 12).

CONTRIBUTIONS  
TO PATHOLOGY  
AND THERAPY

## INTESTINAL OBSTRUCTION.

### III. THE DEFENSIVE MECHANISM OF THE IMMUNIZED ANIMAL AGAINST DUODENAL LOOP POISON.\*

BY G. H. WHIPPLE, M.D., H. B. STONE, M.D., AND B. M. BERNHEIM, M.D.

*(From the Hunterian Laboratory of Experimental Pathology, Johns Hopkins Medical School, Baltimore.)*

In previous communications (1, 2, 3) we pointed out the value of closed duodenal or intestinal loops in the study of some of the obscure features of intestinal obstruction. The closed loop may be likened to a volvulus in which there has been no vascular disturbance or obstruction, and the clinical picture in the two conditions may be identical. The closed loop usually employed in our work is produced in dogs by heavy ligatures placed just below the pancreatic duct and again just beyond the duodenojejunal junction combined with a posterior gastro-enterostomy. The intoxication which develops from the condition must be limited to two factors, bacterial growth and epithelial activity, as other factors such as gastric and pancreatic juice, bile, and products of food digestion are excluded.

Our published experiments show that dogs with closed duodenal loops will die in about two days from acute intoxication, and further that a substance can be isolated from the closed loops, which, if injected into a normal dog, will cause similar but more intense signs of intoxication. The picture is one of severe and fatal shock,—low blood pressure and temperature, vomiting and diarrhea with extreme splanchnic congestion. Moreover, this toxic substance can be extracted from the mucosa of the closed duodenal loops and destruction of the mucosa prevents its appearance in the loops, indicating that the epithelium is essential to its production. The loop in question can be excised without any intoxication resulting therefrom.

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Some of our early experiments led to the conclusion that a normal dog which had been injected intravenously with a sublethal dose of this toxic material became relatively immune, and could withstand much larger doses given by subsequent injection with little intoxication. This immunity can be demonstrated with little difficulty in spite of the varying reaction of normal dogs to the toxic material obtained from the duodenal loops. It was also noted that healthy dogs immunized by injection of the toxic substance can survive operation and the production of a closed duodenal loop for a period of six days or longer. The average duration of life in a non-immune dog after production of a closed duodenal loop is two days, the maximum four days. Further, the immune dogs do not show the early signs of intoxication which are so evident in the untreated dogs.

It seemed likely that this immunity might be explained by the presence of the protective ferments which appear in the blood after the injection of proteids and peptones (Abderhalden (4)). Repeated injection of sublethal doses of the poisonous material from duodenal loops showed that the blood after a suitable period was free from any ferments capable of dissolving various peptones as tested by means of the polariscopic method.<sup>1</sup> The material injected was obtained from closed loops, digested for many days at 38° C., heated to 70° C. for one half hour, centrifugalized, and filtered (Gooch crucible). It seems clear that the cleavage had been sufficient to destroy all peptones. Additional evidence is supplied by the observation that guinea pigs cannot be sensitized by the material so that the anaphylactic reaction may later be elicited. Guinea pigs sensitized by the blood of a closed loop dog will not react to the duodenal loop fluid prepared as outlined above.

Evidence is submitted below to show that the poison found in the closed loop of the dog or cat, and in the human intestine in acute obstruction, may be the same. The poisons have the same toxic effects and may all be used to immunize dogs against subsequent lethal doses of the material from a closed duodenal loop. Further it is noted that a dog (No. O-66) which recovered from a simple obstruction was extremely resistant to the intoxication of a closed

<sup>1</sup> These observations were made by Dr. E. K. Marshall.

duodenal loop, exactly like a dog which had been immunized with loop poison. This fact surely indicates that a poison is absorbed under these conditions, and also that the poison present in simple obstruction and in the closed loop may be identical.

The poison found in the closed loops is a resistant body (1). It is evident that the immune dog develops new power or increases its normal capacity to destroy this poison. The experiments below show that the immune serum is inert but that the protective agent resides in the organ cells and extracts. The liver, spleen, and lungs are very active in destroying the duodenal loop poison when a mixture is incubated for a few hours or days. The clear filtrate from the digested immune liver can destroy the poison after autolysis for hours, indicating possibly some ferment as the active agent.

Normal tissues act on the poison very slowly, and weeks may be required to show any definite destruction of the toxin. Normal liver acts more quickly than other normal organs, but is far weaker than the immune liver. This fact may be taken as evidence of the method of defense of the body against this poison. This protective body or ferment is not a stable factor, but may disappear; so too the acquired immunity of dogs may vanish after a period of weeks or months.

#### METHOD.

The method will be given here in outline; the full account will be found in previous publications. The material obtained from the duodenal loop is allowed to undergo autolysis with chloroform and toluol for several days at 38° C., after which it is heated at 60° to 70° C. for one half hour or longer, centrifuged at high speed for several minutes, and the supernatant fluid filtered through filter paper or asbestos (Gooch crucible). The filtrate may be faintly acid or alkaline. Unless otherwise stated the material obtained from the intestinal loops or mucosa is treated in this manner before injection. The operative technique has been described and illustrated in a former publication. It was found that intraperitoneal injection of this material caused some inflammation and that subsequent operation was not infrequently followed by peritonitis, resulting from the slight soiling of the peritoneum during the production of the gastro-enterostomy. Intravenous injection is much more satisfactory when it is desired to produce an immunity.

#### EXPERIMENTAL OBSERVATIONS.

##### DOG IMMUNIZED TO DUODENAL LOOP FLUID. LETHAL DOSE. SURVIVED.

*Dog O-31.—Mongrel hound, male.*

Jan. 29, 1913. 12 M. Intravenous injection of 20 c.c. of duodenal fluid (dog

O-12). 1 P. M. Muscular tremors and intoxication but no vomiting. 3 P. M. Repeated attacks of vomiting. Dog looks rather sick.

Jan. 30. Dog appears normal.

Feb. 1. 2 P. M. Intravenous injection of 15 c.c. of duodenal fluid (dog S-51-54). This duodenal fluid, 20 c.c. in amount, was fatal to a 15 pound dog in four and one half hours. 4 P. M. Repeated vomiting and much fluid diarrhea.

Feb. 2. Dog has recovered completely and has a good appetite.

Feb. 4. Dog in good condition; weight 22½ pounds. 3 P. M. Intravenous injection of 20 c.c. of duodenal fluid (dog S-51-54). 3.30 P. M. Vomiting and much fluid feces. 4.30 P. M. Dog improving. Pulse is much stronger; no vomiting.

Feb. 6. Dog is in good condition; weight 21¼ pounds. 10.30 A. M. Temperature 38.2° C. Ether anesthesia and kymograph observation. Intravenous injection of duodenal fluid, 30 c.c. (dog O-26). This fluid, 30 c.c., was fatal in seven hours to a dog weighing 25 pounds. The kymograph record (dog O-31) shows initial drop in blood pressure as usual, followed by a slow secondary drop always seen in fatal poisoning. At the end of the kymograph record the temperature was 37.3° C. Following recovery from ether, vomiting and diarrhea were marked. 12.30 P. M. Temperature 39.6° C. 2.30 P. M. Dog has vomited and had some dark fluid feces; appears much intoxicated but can walk about. 4 P. M. Temperature 39.7° C.

Feb. 7, 10 A. M. Dog is rather sick, but this is due to an extensive phlegmon developing about the neck wound involving the region of the shoulder. Temperature 39° C. 12 M. Bled from carotid. Ether anesthesia.

*Autopsy.*—Performed at once. There is an extensive area of inflammation at the root of the neck. Thorax and lungs negative. Liver rather pale. Gastro-intestinal tract normal. Blood serum from this dog used below to show the absence of ferment capable of destroying duodenal poison. This serum together with a normal control showed no ferment activity after digestion with peptone and gelatin, with the polaroscopic method (Abderhalden).

#### INCREASED RESISTANCE TO DUODENAL LOOP FLUID.

Dog D-38.—Small pup; weight 10 pounds.

Jan. 16. Intravenous injection of duodenal loop fluid from a cat. This caused considerable intoxication, vomiting but no diarrhea.

Jan. 23. Dog is noisy and active.

Jan. 24, 10 A. M. Duodenal fluid, 20 c.c. (dog O-11), given intraperitoneally. 12 M. Frothy vomitus below cage, but no diarrhea.

Jan. 25. Dog is better, but abdomen is tender.

Jan. 28. Dog in good condition; weight 10 pounds. 10.30 A. M. The duodenal fluid was standardized. 20 c.c. killed a dog weighing 10½ pounds in four hours, with typical signs of acute intoxication, the experiment being done at the same time with this experiment. A kymograph record (dog D-38) was taken during this intravenous injection of 32 c.c., which is a 50 per cent. increase above the known lethal dose. The fluid caused a profound drop in blood pressure followed by a slow rise and slowing of pulse.

12.30 P. M. Dog badly shocked. Passes a little fluid feces. Pulse just palpable. Respiration slow; appears fatally poisoned. 3 P. M. Dog is badly shocked

but has improved slightly, and the diarrhea has ceased. 4 p. m. Passes a large amount of fluid feces rich in mucus and bile. Bile-stained vomitus abundant. Pulse is much stronger. 6 p. m. Condition about the same.

Jan. 29, 9 a. m. Found dead.

*Autopsy.*—Beginning peritonitis over a large intussusception involving the middle portion of the small bowel. Thorax negative. Viscera are not much congested. Jejunum contains a good deal of fluid. The mucosa is perhaps slightly pinker than normally. It is probable that this dog would have survived this large dose of toxic material but for the intestinal intussusception, brought on undoubtedly during the active peristalsis which always follows the injection of this poisonous material.

The preceding experiments (dogs O-31 and D-38) show that a considerable grade of immunity may be established against the loop poison even by a single sublethal dose. Other experiments given below bring out the same point (dog O-51). The following experiment (dog O-43) shows again the increasing immunity following sublethal doses of the poison; and it appears that it is transient and may drop to a low level or disappear completely after a period of many weeks.

#### IMMUNITY TO DUODENAL LOOP FLUID DISAPPEARS IN THREE MONTHS.

*Dog O-43.*—Strong fox-terrier, male; weight 25½ pounds.

Feb. 17, 11 a. m. Duodenal fluid, 10 c.c., given intravenously (dog O-33-34). 12 m. Dog looks shocked. 2 p. m. No diarrhea; no vomitus. Dog improving.

Mar. 19, 11 a. m. Duodenal fluid (X), 14 c.c., given intravenously. 10 c.c. of this fluid had poisoned fatally within four hours a normal dog weighing 25 pounds. 4 p. m. Much vomiting and profuse diarrhea. The fluid stools are rich in mucus, as seen in fatal poisoning. 5 p. m. Pulse is weak, but dog is evidently recovering.

Mar. 20. Dog has recovered completely.

Mar. 24, 12 m. Duodenal fluid, 20 c.c., given intravenously (dogs O-33 and O-34). 14 c.c. of the fluid poisoned a dog fatally in six hours (dog O-38; weight 21 pounds). 3 p. m. Dog has vomited repeatedly. Much fluid diarrhea, but does not look badly shocked. 5 p. m. Diarrhea continues with tenesmus and bloody mucus. Animal looks sick.

Mar. 29. Dog in normal condition.

Apr. 12. Dog in fairly good condition; weight 22 pounds. 12 m. Duodenal fluid (X), 14 c.c., given intravenously. 2 p. m. Dog has had a little diarrhea; no vomiting. Pulse fair. 8 p. m. Dog eats and looks well. Pulse fair. 8 p. m. Dog eats and looks well. Pulse normal. The poisoning is obviously much less marked at this time than in the previous month.

July 25. Dog has mange, but is in fair condition; weight 22.5 pounds. 11 a. m. Dog given a lethal dose of duodenal fluid, 150 c.c. (dog O-52). This fluid had been boiled and filtered and tested on a normal dog (No. O-101), weighing

24 pounds. The same amount of fluid used, 150 c.c., caused in the control dog fatal intoxication in eight hours with typical findings of an acute intoxication. 12.30 P. M. Dog has had one fluid stool and shows a slow deep respiration. 2.30 P. M. Dog is severely shocked and more soft feces are passed. 5 P. M. Diarrhea continued. Dog is very sick and died shortly after this observation.

*Autopsy.*—July 26, 9 A. M. Heart contained blood clots. Lungs show a great deal of edema. Liver, spleen, and stomach normal. Duodenum shows a mottled pink and red mucosa. Ileum mucosa is pale and the small bowel contains a great deal of fluid feces. Kidneys show a moderate grade of chronic nephritis. The resistance of this dog to duodenal poisoning at this time was not above normal and very much less than during the period three months earlier, when lethal doses of poison were survived with relatively little intoxication.

#### IMMUNITY WITH DOG AND HUMAN MATERIAL.

Dog O-90.—Black and tan male; weight 10 $\frac{3}{4}$  pounds.

May 24. Duodenal fluid, 50 c.c., (dog O-77) given intravenously. This caused some vomiting and diarrhea, but the dog made a good recovery.

June 24. Weight 11 pounds. 3 P. M. Human obstruction fluid (3921), 50 c.c., given intravenously. This did not cause severe intoxication, and it was slightly toxic to normal dogs.

June 25, 9 A. M. Dog is active and lively.

June 26, 12 M. Dog is in good condition; weight 10 $\frac{3}{4}$  pounds. Human obstruction fluid (jejunum 3759), 30 c.c., given intravenously. This fluid was very toxic, 30 c.c. being fatal to dog O-82 in five hours. Weight 13 $\frac{1}{4}$  pounds. 4 P. M. A few soft stools but no diarrhea. No vomiting. No evidence of shock.

June 27. Dog is active and hungry.

The preceding experiment (dog O-90) and one cited below (dog S-90), as well as other observations, show that the poisonous material present in the closed duodenal loops is similar to that present in human intestinal obstruction. Examples need not be multiplied but it can be stated that animals can be immunized against the duodenal loop poison of their own species (*e. g.* dog) by the material obtained from the closed loops of other species (*e. g.* cat), or from intestinal obstruction in man.

#### INTESTINAL OBSTRUCTION RELIEVED GIVES IMMUNITY TO CLOSED DUODENAL LOOP FLUID.

Dog O-66.—Yellow mongrel, male; weight 14 pounds.

Mar. 28. Ether anesthesia and operation; simple intestinal obstruction. Small intestine closed by ligature midway between the pylorus and ileocecal valve.

Mar. 31. Dog is dull and sick.

Apr. 1. Intravenous injection of a mixture containing ascitic fluid and organ extracts from immune dog.

Apr. 3. Dog seems slightly better.

Apr. 4. Dog has passed feces and is obviously improving. The ligature has probably cut through and established the continuity of the intestinal tract.

Apr. 5. Dog has had diarrhea and appears in good condition.

Apr. 10. Dog appears normal; weight 13 $\frac{3}{4}$  pounds.

Apr. 11. Exploratory operation under ether anesthesia. The ligature has cut through, and the lumen of the small gut was practically normal at this point but the intestine above the site of ligation is definitely dilated and its walls are hypertrophied. A closed duodenal loop was done in the usual manner, together with a posterior gastro-enterostomy.

Apr. 12. Dog shows no evidence of intoxication.

Apr. 14. Weight 11 $\frac{1}{4}$  pounds. No signs of acute intoxication.

Apr. 15. Dog has distemper with purulent discharge from nose. Pulse is good and strong. Weight 11 $\frac{1}{4}$  pounds. 4 p. m. Given intravenously ascitic fluid, 500 c.c., from dog 12-44 (experimental passive congestion).

Apr. 16. Dog appears fairly well and walks about. Temperature 39.1° C. Weight 10 $\frac{1}{2}$  pounds.

Apr. 17. Dog eats a little food and is stronger. No evidence of intoxication. Weight 10 $\frac{1}{2}$  pounds.

Apr. 18. Dog is in fair condition; weight 10 $\frac{3}{4}$  pounds.

Apr. 19. Dog is improving and passed a solid stool. Weight 12 pounds.

Apr. 21. Condition remains the same. There is evident distemper. Temperature 39.1° C. Weight 12 pounds.

Apr. 22, 11 a. m. Weight 12 $\frac{1}{4}$  pounds. Ether anesthesia, exploratory laparotomy. The upper ligature had cut through, and the lower ligature was intact. The lumen here was completely obstructed. Gastro-enterostomy perfect. The loop was decidedly thickened and dilated, but contained only 30 to 50 c.c. (estimated). The duodenal loop was again isolated by means of cutting across the bowel and inverting the ends, as has been described in previous papers. Such dogs died as a rule within twenty-four hours and rarely survived thirty-six hours.

Apr. 23, 3 p. m. Dog curled up quietly. Weight 11 $\frac{1}{2}$  pounds. Temperature 39.3° C.

Apr. 24, 12 m. Dog looks sick. Pulse is weak. Temperature 38.1° C. Weight 11 $\frac{1}{2}$  pounds. 5 p. m. Died.

*Autopsy.*—Performed at once. The peritoneal cavity contains 200 c.c. of purulent, foul smelling fluid. There was an abscess at the lower end of the duodenal loop which had ruptured, causing general peritonitis. Thorax, heart, lungs, spleen, and liver negative. Stomach collapsed. Small intestine collapsed and contracted tightly around the root of the mesentery. The loop is small and contains thick, tenacious paste. Its mucosa, except for that covering the inverted ends, is normal.

The preceding experiment (dog O-66) is worthy of careful study and analysis. A simple obstruction of the small intestine presented the usual picture of intoxication, which was somewhat relieved by an injection of a solution of organ extracts from an immune dog. We shall not discuss this point here, and merely state that by about the fifth or sixth day there was improvement. It

was obvious that the obstruction had been relieved by the cutting through of the ligatures.

An exploratory operation two weeks after the first operation showed that the obstruction had been completely relieved, but the jejunum was dilated and hypertrophied. It may be supposed that this dog had been immunized by absorption from his own mucosa during the simple obstruction.

At this time a closed duodenal loop was made as usual and it is noteworthy that the dog appeared in no way intoxicated during the next few days, although he received no fluid infusions and no treatment of any kind, except on the fourth day. The closed loop was present for eleven days, and at this time a second exploratory operation showed an enlarged and thickened duodenal loop containing fluid. The lower ligature was intact and the upper had cut through allowing the escape of fluid on the application of pressure. In rare instances in very vigorous dogs we have observed the cutting out of a ligature with leakage of the loop fluid into the bowel above or below. These animals die, but may survive four or five days. The loop in this dog was securely closed the first few days and later became partly drained. It should be recalled that the majority of untreated dogs with duodenal loops draining externally die in three to six days.

There is no escape from the conclusion that this dog had a strong immunity toward the intoxication resulting from a closed duodenal loop, and the immunity developed as a result of a preceding simple intestinal obstruction, which emphasizes the fact that the intoxication of simple obstruction is similar or probably identical with that developing in association with a closed duodenal loop.

**IMMUNIZED DOG WITH CLOSED DUODENAL LOOP. KILLED AFTER SIX DAYS.**

*Dog S-51.*—Strong adult male; weight 15 pounds.

May 14. Dog was given intraperitoneally 30 c.c. of material obtained from the closed duodenal loop mucosa of dog S-29. This caused very little intoxication.

May 15. Dog seems normal.

May 17. 11 A. M. Dog was given 28 c.c. of material obtained from a closed duodenal loop (dog S-38). This likewise gave little indication of intoxication.

May 18-21. Dog is normal and eats well.

May 22, 12 M. Dog was given intraperitoneally duodenal loop fluid, 30 c.c. of clear filtrate, prepared as usual, obtained from dog S-11. 2 P. M. Dog had some diarrhea but no vomiting.

May 29, 3 P. M. Ether anesthesia and operation as usual with isolation of closed duodenal loop.

May 30, 1 P. M. Temperature 38° C. Dog eats; no vomiting and no diarrhea.

May 31, 10 A. M. Temperature 38.8° C. Dog eats well and seems in excellent condition. 5 P. M. Temperature 39° C. Dog refused food.

June 1, 10 A. M. No vomiting and no diarrhea, but refused food. Temperature 39° C. Given milk by stomach tube. 5 P. M. Temperature 38.6° C. Condition about the same.

June 2, 12 M. Dog has considerable diarrhea and drinks water eagerly; pulse good. Temperature 37° C. Given milk again by stomach tube, which was followed by vomiting. Dog has lost considerable weight and strength.

June 3, 10 A. M. Dog lies quietly in the cage. Temperature 36° C. Dog has had some diarrhea, the fluid being streaked with a little blood. Pulse is slow but of good volume and tension. 2 P. M. Temperature 35.5° C. Given 200 c.c. of salt solution subcutaneously, as animal will not retain fluids given by the stomach.

June 4, 10 A. M. Dog appears improved. Temperature 37.5° C. The pulse is strong and full. No diarrhea, and stools are pasty. 12 M. Given milk by stomach tube. 2.30 P. M. Pulse strong. Temperature 37° C. Given ether and bled from carotid. The blood pressure was good at this time. Blood showed a good deal of hydremia, giving about four fifths by volume of serum after centrifugalization. The dry weight of the blood at this time was 12.4 and 12.3 per cent., in parallel determinations.

*Autopsy.*—Performed at once. Heart, lungs, thorax, and peritoneal cavity normal. Spleen is pale and fibrous. Pancreas and kidneys are normal. The stomach contains bile-stained fluid and milky curds. The mucosa is normal. Small intestine shows a pale normal mucosa, in which hookworms are rather numerous. The duodenal loop is large and flabby. Its ends are closed tightly and it contains about 150 c.c. of canary yellow, purulent material. The mucosa is slightly swollen and pinkish, but intact throughout. Three hookworms are alive and active and adherent to the mucosa.

*Microscopical Examination.*—Spleen is atrophic. Kidneys, liver, duodenal loop, jejunum, and stomach mucosa are all normal.

#### IMMUNIZED DOG WITH CLOSED DUODENAL LOOP. KILLED AFTER FOUR DAYS.

Dog S-90.—Old mongrel, female; weight 12½ pounds.

July 16, 3 P. M. Ether anesthesia and intravenous injection of fluid obtained from a case of human intestinal obstruction. This material had undergone autolysis in the usual way for several days, was heated at 60° to 70° C. for two hours, centrifuged, and filtered. 20 c.c. of this broth-like filtrate were given intravenously and the ether removed at once. The duration of the anesthesia was only about ten minutes. 5 P. M. Pulse is just palpable and dog appears much shocked. Dog has vomited twice and passed one semifluid stool. 8.30 P. M. Pulse is good and dog is much improved. No diarrhea.

July 17, 9 A. M. Dog will not eat.

July 18, 10 A. M. Dog appears rather sick; in the afternoon ate some food and seems better.

July 21. Dog seems well.

July 22, 3 p. m. Ether anesthesia and operation as usual with isolation of a closed unwashed duodenal loop.

July 23, 9 a. m. Temperature 39° C. Dog is active. 5 p. m. Temperature 38.9° C.

July 24, 2 p. m. Rectal temperature 38.4° C. Dog drinks water but will not eat.

July 25, 1 p. m. Rectal temperature 38.4° C. Dog has lost weight and strength and refuses food.

July 26, 10 a. m. Rectal temperature 38.7° C. Pulse is good. 3 p. m. Rectal temperature 38.6° C. Dog is fairly strong, but has lost a good deal of weight. Given ether and bled from carotid. The pulse pressure is strong, and there is no indication of a severe intoxication. It is probable that this animal would have lived one or two days longer.

*Autopsy.*—Performed at once. Peritoneal cavity, thorax, heart, and lungs are all normal. Spleen and kidneys are normal. Liver is pale brownish in color, but otherwise normal. The duodenal loop is not distended. The ends are tightly closed. It contains about 10 c.c. of pasty, creamy material, which sets into a sort of jelly on contact with water. This is squeezed out and the mucosa scraped off as usual. The mucosa is normal and intact throughout. There are no ulcers and no submucous hemorrhages. Stomach and duodenum above the ligature contain bile-stained fluid. The mucosa is pale and intact. The small intestine contains bile-stained fluid, and its mucosa is pale and intact. The gut is uniformly constricted throughout.

The two preceding experiments (dogs S-51 and S-90) show a type of immunity which develops following the injection of the toxic material in sublethal doses. The results are the same whether material from closed loops in dogs or from intestinal obstruction in man is used to immunize the animals. The dogs with the closed loops do not show the usual acute signs of intoxication seen in dogs not previously injected. They survive often far beyond the maximum period for untreated dogs with closed loops. Hence the poison found in the closed loops is the cause of the intoxication resulting from their experimental production. A similar poison is found in human intestinal obstruction and presumably is concerned with the intoxication found in this condition.

The two next experiments (dogs S-72 and S-70) illustrate some of the difficulties in the work with the closed-loop dogs. The immunity following repeated doses of the poison will protect effectively against the loop intoxication, but not against peritonitis. The closed loops may behave very differently under various conditions. At times in a fatal case the loop will be found collapsed and containing only a few cubic centimeters of thick, pasty material. These cases are favorable for immunity work. If an immunity is

established in such a dog the animal may live more than double the usual period, but in most animals there is a rapid accumulation of fluid in the loop which produces tension and favors injury and ulceration of the mucosa which may effect a perforation. Perforation, of course, causes peritonitis with rapid death, and adds a confusing factor to the picture.

**IMMUNIZED DOGS WITH CLOSED DUODENAL LOOP. DEATH IN FOUR DAYS WITH PERITONITIS.**

*Dog S-72.*—Strong adult fox-terrier, male; weight 22 pounds.

June 21, 11 A. M. Dog was given 22 c.c. of duodenal loop fluid obtained and prepared as usual (dog S-65). This was injected intraperitoneally and caused no pain. 1 P. M. Dog has vomited once, but there is no diarrhea.

June 22. Dog is perfectly normal and eats well.

June 24, 5 P. M. Dog given intraperitoneally 25 c.c. of duodenal fluid (dog S-51).

June 25, 9 A. M. Dog is normal.

June 27, 3 P. M. Ether anesthesia and operation with isolation of closed duodenal loop in the usual way with washing out of contents of loop.

June 28. Dog is quiet and refuses food.

June 29, 10 A. M. Dog is in good condition. Pulse strong. Rectal temperature 38.5° C. Muscular tremors are noted. 4 P. M. Temperature 39° C. Given one half pint of milk. 5 P. M. Dog has vomited milk and bile-stained material. Small amount of soft blood-tinged feces are passed. Dog seems obviously intoxicated.

June 30, 12 M. Dog again given milk with one egg by stomach tube. Weight 19 pounds. Rectal temperature 40° C. Pulse strong and regular. 1 P. M. Dog vomited bile-stained coagulum.

July 1, 9 A. M. Condition about the same. Temperature 39° C. 11 A. M. Dog is given milk which is vomited at once, and later a little water which is also vomited immediately. 2 P. M. Pulse is slow and weak. Temperature 38.8° C.

2.15 P. M. Death. Blood obtained a few minutes after death showed dried weight of 22.3 and 22.4 per cent, in parallel determinations.

*Autopsy.*—Performed at once. Thorax, heart, lungs, and spleen are all normal. The peritoneal cavity contains some purulent, turbid fluid, about 20 c.c. in amount. The serous surfaces are injected and covered with tiny ecchymoses. It is found that there had been leakage from the lower puncture wound and lower ligature which had cut into the gut wall. Jejunum shows a mottled, pinkish mucosa with little injection. The ileum is quite normal and contains soft yellow feces, and fecal material slightly stained with blood. Liver is considerably injected. Kidneys and stomach are normal. The loop contains about 20 c.c. of slate-colored, thick material having a fecal odor. The mucosa is intact throughout and normal except for a little injection.

*Dog S-70.*—Small pup, male; weight 11 pounds.

June 20, 11.30 A. M. Ether anesthesia with kymograph observation. The material was obtained from the intestinal mucosa (dog S-66), a case of simple

intestinal obstruction. The intestinal mucosa, washed and scraped off, was allowed to undergo autolysis for six days, heated at 65° C. for one hour, centrifugalized, and filtered. 55 c.c. of this clear filtrate caused the usual drop of the blood pressure with return to normal. 12.20 P. M. Dog removed from kymograph. 1 P. M. Dog passed a semifluid stool. 2.30 P. M. Diarrhea is marked and dog severely shocked. Pulse is fair. 5 P. M. Dog is weak. Diarrhea persists.

June 21, 9 A. M. Dog seems normal and eats vigorously.

June 23. Condition is normal.

June 24, 12 M. Dog is normal; weight 11½ pounds. 12.30 P. M. Ether anesthesia and kymograph observation. The material was obtained from dog S-46 (drained duodenal loop). 90 c.c. of this material was given intravenously, causing an initial drop of blood pressure followed by a rise above normal. 1 P. M. Dog removed from kymograph. 3 P. M. Dog is much shocked: vomiting and diarrhea are marked. 5 P. M. Dog is quite sick. Muscle tremors are marked. Pulse is fair, and diarrhea continued.

June 25, 9 A. M. Dog seems well.

July 11, 3 P. M. Dog in excellent condition. Ether anesthesia and operation as usual, with isolation of closed unwashed duodenal loop.

July 12, 9 A. M. Dog seems well. 4 P. M. Condition the same.

July 13, 9 A. M. Dog has vomited. Rectal temperature 38.5° C. Pulse is strong. 4 P. M. Dog has passed solid and semifluid feces. 9 P. M. Pulse regular and strong.

July 14, 10 A. M. Dog seems pretty well. Temperature 38.3° C. Pulse regular, but tension is poor. Vomitus is present under the cage, and partly digested food is present in it. 5 P. M. Dog is very weak and toxic. Pulse is of low tension. Given 200 c.c. of water and vomited immediately. Temperature 38.2° C.

July 15, 9 A. M. Pup is very toxic. Pulse is weak. Rectal temperature 39.4° C. Vomited at once water given by stomach tube. Muscle tremors are present. 9.30 A. M. Death.

*Autopsy.*—Performed at once. Thorax and heart are normal. Lungs show a few small patches of consolidation along the edges of the left lower lobe. Spleen is negative. Liver is congested. The peritoneal cavity contains a little thin, purulent material, about 5 c.c. in amount. The surfaces are injected and show scattered ecchymoses. The leak from which the peritonitis developed is about the upper ligature where a tiny perforation and pocket of pus is found. The loop is not distended and contains only 20 c.c. of rather thick fluid. The mucosa is quite intact, slightly injected, and shows no ulcers and no hemorrhage. The stomach contains bile-stained fluid. The jejunum shows a pinkish red mucosa, with definite injection of the villi. Considerable fluid is present. The ileum shows a very faint pinkish mucosa. The large intestine and kidneys are normal.

#### IMMUNE SERUM INACTIVE TO DUODENAL LOOP POISON.

*Dog O-31.*—Mongrel hound; weight 21 pounds. This dog (see history above) was given repeated injections of duodenal loop fluid and survived a known lethal dose. The blood after defibrination was centrifugalized and 65 c.c. of blood serum were obtained. The red blood cells were then washed with salt solution, centrifugalized, and the washings added to the serum, making 130 c.c. in all.

To this was added duodenal loop fluid (dog O-26), 30 c.c., an amount which was known to be a lethal dose as tested on other animals. This mixture of immune dog serum and duodenal fluid together with chloroform and toluol was incubated for twenty hours at 38° C., and after heating and filtration was injected intravenously into dog O-37, a mongrel Irish terrier, male, weighing 13½ pounds.

Feb. 2, 9 A. M. Ether anesthesia and kymograph record during injection. There was no immediate depression of blood pressure and obviously the incubation with serum neutralizes or destroys this substance, causing the acute primary drop in blood pressure. There was a remarkable secondary fall in blood pressure coming on after about twenty minutes accompanied by great slowing of pulse. The dog passed fluid feces at the end of fifteen minutes and vomited before coming out of the anesthesia. 1.30 P. M. Death.

*Autopsy.*—The findings are in every respect typical of acute poisoning by the duodenal loop fluid.

#### IMMUNE SERUM DOES NOT DESTROY DUODENAL LOOP POISON.

*Dog 12-34.*—Small pup; weight 9 pounds.

Jan. 9. Intravenous injection of material from closed intestinal loop of cat. This caused a moderate grade of intoxication with diarrhea.

Jan. 14. Complete recovery. Ether anesthesia and bleeding from carotid. The blood serum gave negative results for the ferments described by Abderhalden, with peptone and the polariscope method. The remaining blood was then defibrinated and 70 c.c. of serum were obtained.

*Autopsy.*—Normal organs throughout. Blood, 70 c.c., plus duodenal fluid (dog O-11), 50 c.c., were placed in the incubator with toluol and chloroform for twenty-four hours at 39° C. There was slight turbidity at the end of this time. The fluid was heated to 55° C. for one half hour, centrifugized, and filtered. This mixture, 115 c.c., was given intravenously to dog O-23, a strong adult fox terrier, weighing 16 pounds. 10.30 A. M. Ether anesthesia and kymograph record during the entire period of injection. There was no sudden drop in blood pressure and no marked drop during the period of observation (one half hour). A well marked erythema of the skin appeared over the legs and abdomen. 2 P. M. Dog is prostrated and has vomited. 3.30 P. M. Death.

*Autopsy.*—The findings were typical of acute poisoning by duodenal loop fluid, described and illustrated in previous publications (2).

*Dog O-22.*—Small pup; weight 7 pounds.

Jan. 11, 12 M. Human mucosa (3782). Autolysis for twelve weeks. Heated at 95° C. for thirty minutes; no precipitate; filtered. 95 c.c. intravenously gave little evidence of severe intoxication. 2.30 P. M. Dog appears fairly well. No diarrhea.

Jan. 12. Dog is normal.

Jan. 20. Bled for ferment tests. The Abderhalden tests with this blood serum were negative, as above, for the presence of protective ferments. Blood serum, 50 c.c., was combined with blood serum of dog 12-30 to be used later.

*Dog 12-30.*—Fox-terrier, male; weight 14½ pounds.

Jan. 11. Human intestinal contents treated as above (dog O-22). 40 c.c. given intravenously caused little reaction.

Jan. 12. Dog is normal.

Jan. 20. Ether anesthesia and bleeding. This blood serum was tested with negative result for the Abderhalden ferments. The blood serum (110 c.c.) combined with that of dog O-22 (50 c.c.) was added to duodenal loop fluid of dog O-11 (50 c.c.). This mixture was incubated together with chloroform and toluol for 22 hours at 39° C., then heated for one half hour at 60° C., and filtered. This clear brown fluid, 205 c.c., was given intravenously to dog O-27, a fox terrier, weighing 12 pounds.

*Dog O-27.*—Jan. 21, 12 m. Ether anesthesia. Kymograph observation showed a slow transient drop during injection, with a tendency to return to normal within thirty minutes. 3 P. M. Pulse is good. No evidence of shock. Has passed one solid stool. 8 P. M. Found dead, but quite warm. Fluid feces and vomitus abundant under cage.

*Autopsy.*—Performed at once. Heart still fibrillating. Blood clots slowly. Lungs show patches of hemorrhagic edema. Spleen is greatly enlarged. Liver is huge and deep purple in color, and very tense.

Mesenteric vessels are greatly dilated. Stomach contains a little fluid and shows a pale mucosa. Duodenum shows engorgement of the villi, but is contracted. The small intestine contains much blood-tinged fluid. The mucosa is not deep purple, but is mottled pink and red.

The three preceding experiments (dogs O-31, 12-34, and 12-30) show that the serum of dogs immunized by sublethal doses of the poison will not neutralize or destroy the duodenal loop poison. This contrasts strikingly with the observations given below dealing with immune organs and organ extracts which can destroy this poison when the mixture is incubated for a short space of time. The protective ferments described by Abderhalden play no part in this reaction and were absent in all cases examined.

#### LIVER, SPLEEN, AND LUNGS OF IMMUNE DOG DESTROY DUODENAL LOOP POISON.

*Dog O-51.*—Mongrel female; weight 15½ pounds.

Mar. 15. Duodenal loop fluid digested with mucosa of immune dog. The filtrate was given intravenously with little evidence of intoxication.

Mar. 16. Dog is normal.

Mar. 19. Duodenal fluid (X), 20 c.c., given intravenously. This caused a little vomiting and a slight amount of diarrhea, but no grave intoxication. The dog has evidently a considerable grade of resistance as this amount of fluid contains more than enough toxin to kill a normal dog weighing 25 pounds.

Mar. 20. Dog rather weak, but is recovering.

Mar. 22. Dog is normal, and eats as usual. Weight 13 pounds. Ether anesthesia, bleeding, and perfusion with 0.9 per cent. salt solution to wash out all the blood from the organs which are normal in all respects. The following extracts and emulsions were made with the addition of a standardized duodenal loop fluid, of which 10 c.c. was fatal to a normal dog weighing 25 pounds in a period of four hours.

(1) Duodenal loop fluid (X), 20 c.c., plus washed mucosa from small intestine, 50 c.c. Dilution with water, toluol, and chloroform; autolysis for five days; heated at 60° C. for one hour. This fluid mixture caused fatal and typical intoxication in a period of ten hours (dog O-64 below). It is probable that some of the poison was destroyed.

(2) Duodenal fluid (X), 20 c.c., plus whole spleen ground up with sand and made up to a thin emulsion with water, toluol, and chloroform. Autolysis for three days. Heated at 60° C. for two hours followed by centrifugalization. This fluid mixture caused no acute intoxication (dog O-63 below) and the poison had been destroyed.

(3) Duodenal loop fluid (X) plus liver, 250 gm., ground up with sand, extracted in Buchner press, and about 80 c.c. of the thick juice obtained. This was diluted to 190 c.c., centrifugalized, and filtered, giving 160 c.c. of buff colored filtrate. 50 c.c. of this liver juice plus 15 c.c. of duodenal fluid (X) given at once intravenously into a normal dog. The mixture had stood only a few moments before the injection. This caused a fatal and typical intoxication after four hours (dog O-56).

(4) Duodenal fluid (X), 20 c.c., plus liver juice, 50 c.c., prepared as in (3), plus toluol and chloroform. Autolysis for forty-four hours, heated at 60° C. for one half hour, and centrifugalized, giving a clear odorless broth. The entire amount was given intravenously to dog O-57 (see below) and caused no evidence of intoxication. Recovery was immediate, showing complete destruction of poison.

(5) Duodenal fluid (X), 20 c.c., plus both lungs ground to a thin paste with sand, water, chloroform, and toluol. Autolysis for sixty-six hours, heated at 60° C. for one half hour, centrifugalized, and filtered, giving a clear, odorless, pale amber colored fluid. The entire filtrate, 125 c.c. in amount, was given intravenously to dog O-61 with but slight evidence of intoxication.

(1) *Dog O-64.*—Male pup; weight 10½ pounds.

Mar. 27, 12.30 P. M. Intravenous injection of a fluid mixture of immune dog's mucosa digested with duodenal loop fluid. Autolysis for five days and preparation for injection in the usual manner. This caused great and prolonged drop in blood pressure with the appearance of diarrhea within thirty minutes. 4 P. M. Dog is cool and badly shocked. Much fluid feces. 10 P. M. Dog is moribund and cool.

Mar. 28, 9 A. M. Autopsy shows typical picture of acute poisoning with duodenal loop fluid.

(2) *Dog O-63.*—Adult fox-terrier, male; weight 10 pounds.

Mar. 27, 12 M. Intravenous injection of fluid mixture of spleen emulsion from immune dog plus duodenal fluid (X), 20 c.c. Autolysis for three days and preparation as usual. This clear broth, 75 c.c. in amount, gave only a slight drop in blood pressure (kymograph). 4 P. M. One semifluid stool; no vomiting. No evidence of intoxication.

Mar. 28. Dog looks rather sick. No diarrhea. 1 P. M. Dog is quite sick; etherized.

*Autopsy.*—Extensive bronchopneumonia with hemorrhage and edema of both lungs. Liver shows areas of necrosis and hemorrhage. There is only a slight congestion of the intestinal mucosa. It is unlikely that much of the intoxication was due to the duodenal loop poison. Death was probably due in great measure

to the acute pneumonia associated with aspiration of vomitus at the time of anesthesia.

(3) *Dog O-56*.—Fox-terrier, male; weight 14 pounds.

Mar. 22, 4 p. m. Intravenous injection of a fresh mixture of duodenal loop fluid (X), 15 c.c., plus fresh Buchner press liver juice, 50 c.c., of immune dog. Kymograph observation and ether anesthesia. This caused a marked drop in blood pressure with slow recovery. Marked polypnea. 5 p. m. Dog appears greatly shocked. 10 p. m. Found dead and rather cold. Much feces and vomitus below cage.

Mar. 23, 9 a. m. Autopsy showed typical evidence of acute poisoning with duodenal loop fluid. The splanchnic engorgement and congestion are very intense.

(4) *Dog O-57*.—Small bulldog; weight 14½ pounds.

Mar. 24, 10.30 a. m. Intravenous injection of fluid mixture, duodenal loop fluid (X), 20 c.c., plus Buchner press liver juice of immune dog, 50 c.c. Mixture autolyzed for forty-four hours at 38° C., diluted, heated, and treated in the usual way for injection. This clear broth-like, odorless fluid gave a slight drop, followed by rapid return to normal blood pressure (kymograph). 12 m. No vomiting; slight diarrhea. Dog does not appear poisoned.

Mar. 25. Dog is normal.

(5) *Dog O-61*.—Small female; weight 11¾ pounds.

Mar. 25, 12 m. Intravenous injection of fluid mixture, duodenal loop fluid (X), 20 c.c., plus emulsion of lungs from immune dog O-51. Mixture incubated for sixty-six hours at 38° C. and treated in the usual way for injection. This clear fluid, 125 c.c., the total filtrate, caused little reaction on the part of the blood pressure. 2 p. m. Dog is sick, but only one stool is passed.

Mar. 26. Dog is well.

The preceding experiment (dog O-51) brings out interesting points. The dog was immunized by means of a mixture of a standard loop fluid digested with immune mucosa which had destroyed most of the poison; yet the dog developed a high grade of immunity as shown four days later when the animal survived a lethal dose of standard duodenal fluid.

Many of the immune organs were washed free from blood and ground to a paste which was combined with a lethal dose of the duodenal loop fluid, diluted with water to a thin paste, and allowed to autolyze at 38° C. for two to five days with chloroform and toluol. The filtrate was then tested on normal dogs by intravenous injection. The spleen and lung emulsion destroyed the poison. The liver juice (Buchner press), diluted and filtered, also destroyed it rapidly. The intestinal mucosa destroyed some of the poison but a part remained even after five days' digestion. A fresh mixture of liver juice and loop poison gave fatal intoxication, showing that

the reaction is not prompt or does not take place in the blood stream. This result serves as a control. If the protective action resides in a single type of cell, it is the endothelial cell that may be concerned, but it is of course possible that various body cells may develop the reaction or produce the ferment.

**LIVER, SPLEEN, AND MUCOSA OF IMMUNE DOG DESTROY DUODENAL LOOP POISON.**

Dog O-32.—Mongrel, male; weight twenty pounds.

Jan. 30. Duodenal fluid (dog O-12), 15 c.c., given intravenously. This caused vomiting, but no diarrhea.

Feb. 1. Duodenal fluid (dog S-51), 15 c.c., intravenously. This caused vomiting and dog appears somewhat poisoned.

Feb. 4. Dog appears fairly well; weight 18 pounds. Duodenal fluid (dog S-51), 20 c.c., injected intravenously. This caused vomiting but no diarrhea.

Feb. 11, 10 A. M. Duodenal fluid (dog O-26), 26 c.c., given intravenously. 11.30 A. M. Considerable vomitus, but no diarrhea.

Feb. 12. Dog appears quite sick. Temperature 34.2° C. Etherized and bled.

*Autopsy.*—Performed at once. The organs are practically normal. The mucosa of the small intestine is washed and scraped off carefully. The spleen is cut and ground up with sand and mixed with the mucosa.

The spleen-mucosa mixture was then added to 30 c.c. of duodenal loop fluid (dog O-26). This fluid had been tested and was known to be a lethal dose (compare dog O-31) for an animal of 13 pounds weight. This mixture, together with chloroform and toluol diluted to a thick emulsion by water, was incubated at 39° C. for three days. The mixture was then heated at 60° C. for one hour, centrifugalized, but not filtered. The turbid, rather soupy fluid, 100 c.c. in all, was given intravenously to dog O-42, a small female fox-terrier, weighing 11½ pounds. The injection caused a great drop in blood pressure with rapid return to normal after each injection.

Feb. 15, 2.30 P. M. Kymograph. 4 P. M. Dog seems quite well.

Feb. 16. Dog is normal.

The liver obtained from the immune dog O-32, normal in gross, weight about 300 gm., was minced and ground up with sand and combined with duodenal loop fluid (dog O-26), 32 c.c. This was a known lethal dose of loop fluid (dog O-31). The mixture was diluted suitably with 0.9 per cent. salt solution, and then chloroform and toluol were added. This mixture of immune liver tissue and duodenal fluid incubated for twenty-four hours at 40° C. was then centrifugalized, without heating, giving a cherry red, rather turbid fluid. The total amount, 85 c.c., given intravenously had a powerful depressor action (dog O-40, strong female pup; weight 21 pounds).

Feb. 13, 4 P. M. Kymograph. The blood pressure curve returned rapidly to normal and there was no slowing of the pulse. There was remarkable and forcible dyspnea. 6 P. M. Dog appears to be recovering normally from ether. No diarrhea.

Feb. 14. Dog normal in every respect.

The liver residue obtained after centrifugalization and removal of fluid tested above (dog O-40) was carefully saved and digested at 38° C. together with normal pancreas for a period of three weeks. This gave a clear, deep brown

fluid, strongly acid in reaction. It is made faintly alkaline by sodium hydroxide. The fluid was given rapidly intravenously to dog O-45, a small male fox-terrier, weighing 13½ pounds.

Mar. 6, 10 A. M. Ether anesthesia and kymograph. Intravenous injection of total fluid obtained, 270 c.c. This caused a rapid drop in blood pressure with return to normal at the end of thirty minutes. 12 M. No vomiting or diarrhea. Dog is recovering rapidly. There was no abnormality except prolonged oozing from the neck wound, probably associated with antithrombin excess in the blood.

Mar. 7. Dog is normal.

LIVER EXTRACT FILTRATE OF IMMUNE DOG DESTROYS DUODENAL LOOP POISON.

Dog O-55.—Mongrel pup, male; weight 20½ pounds.

Mar. 20, 4 P. M. Intravenous injection of extract made from the mucosa of the drained duodenal loops of dogs O-21 and O-24. The total amount, 125 c.c., caused no immediate change in blood pressure. 8 P. M. Much vomiting and bloody diarrhea. Pulse fair, but animal appears considerably shocked.

Mar. 21. Dog is still quite sick.

Mar. 24. Dog appears well. 12 M. Intravenous injection of 20 c.c. of duodenal loop fluid (dogs O-33 and O-34). This fluid had been standardized on normal dogs. 3 P. M. Dog is quite sick. There is marked salivation and vomiting with profuse diarrhea and severe shock. 5 P. M. Mucous diarrhea and severe intoxication.

Mar. 25. Dog is improving, but still weak.

Mar. 29. Dog in fairly good condition except for infected neck. Ether anesthesia, bleeding from carotid, and washing of organs free from blood with large amounts of normal saline given intravenously during bleeding.

Liver quite bloodless, weight 294 gm., minced and ground up with sand to a thick paste, which was placed in a Buchner press. About 50 c.c. of thick, viscid juice was expressed, and this was diluted to about 130 c.c. This thin, soupy material was allowed to undergo autolysis at 38° C. for sixteen days, giving a clear, deep brown fluid which filtered with ease.

30 c.c. of this clear filtrate were added to 20 c.c. of duodenal fluid (X), a known lethal dose. Autolysis of the mixture for three days gave a very slight turbidity. The material was centrifugalized but not heated, filtered, and injected intravenously, 50 c.c. in amount, into dog O-72, a strong male fox-terrier, weighing about 12 pounds. Kymograph record and ether anesthesia during the period of injection. The filtrate caused a drop in blood pressure followed by rapid recovery. There was no evidence of intoxication, no vomiting, and no diarrhea. The following day the dog was normal in all respects.

The same liver extract (dog O-55), 90 c.c. in amount, was kept in the incubator (38° C.) with chloroform and toluol for a period of sixteen weeks. At the end of this time filtration gave the same clear, whiskey-colored, odorless fluid. This was combined with fresh duodenal fluid, 50 c.c. (dog O-86). This mixture of immune liver juice and duodenal fluid was allowed to remain in the incubator with chloroform and toluol for ten days. At the end of this time the solution filtered with great rapidity. There was only the slightest turbidity, and no odor.

July 31, 12 M. Unheated fluid, 135 c.c., given intravenously to dog O-116, a young female mongrel, weighing 17½ pounds. 3 P. M. Dog greatly shocked. 4 P. M. Death, preceded by vomiting and diarrhea.

*Autopsy.*—Aug. 1. The usual picture following acute intoxication is present. This shows that the ferment capable of destroying the duodenal poison is an unstable body and may disappear from the immune organ extracts after a period of many weeks.

The preceding experiment (dog O-55) adds another important point. The immune liver juice kept in an incubator undergoes autolysis rapidly yielding a clear, whiskey-colored filtrate, which at the end of two weeks contains a ferment capable of rapidly destroying *in vitro* the duodenal loop poison. However, after a period of incubation at 38° C. for sixteen weeks the active body disappeared and the liver extract was inert towards duodenal fluid. This fact should be compared with the observations above (dog O-43), where it is noted that the immunity of an animal will vanish or drop nearly to a normal level during a period of four months.

#### DISCUSSION.

The series of experiments here reported provide, we believe, conclusive evidence that a definite intoxication occurs in animals with closed loops, and that the intoxication is due to absorption of the loop poison. There are good reasons for believing that the intoxication seen in cats and dogs with intestinal loops and in human intestinal obstruction is due to the same poison or a similar one. It is noteworthy that a dog recovering from simple obstruction showed little evidence of intoxication when a closed duodenal loop was established. Other evidence will be brought forward later to show that dogs with simple intestinal obstruction begin to acquire a certain immunity to the intoxication which will protect them against a closed-loop intoxication or the intravenous injection of duodenal loop fluid.

In a recent paper Hartwell (5) criticizes our conclusions and endeavors to minimize the factor of intoxication while emphasizing the loss of fluid as the essential and primary condition. In the conclusions of their first paper Hartwell and Hoguet (6) state: "The above findings indicate that death from intestinal obstruction in dogs results from the presence of toxic substances in the circulating

blood which produce fatal lesions in the kidney, liver and other tissues." In their second paper (7) they say: "The important element, therefore, in the development of the symptoms seen in intestinal obstruction in dogs is the loss of water due to vomiting. The symptoms of intoxication are those resulting from tissue disintegration following this loss."

We have claimed that intoxication is the primary feature and that loss of fluid is secondary to the intoxication, instead of the reverse, as claimed by Hartwell. In his last paper Hartwell (5) admits that there may be intoxication, and so reverts in part to the theory supported by his first paper. He states that intoxication can only appear when there is obvious injury to the intestinal mucosa, and in this we believe he is mistaken, although we admit that given an obvious mucosal injury with ulceration, the intoxication may be more profound. If the theory of injury to the mucosa is the essential factor, why do not all cases of gastro-enterostomy show signs of acute intoxication? Why do not dogs with drained loops where the gut is cut across and drawn into the abdominal wound die sooner than dogs with simple closed loops? Dogs with closed loops may show evidences of grave intoxication at the end of twenty-four hours and yet autopsy may show a collapsed loop with intact mucosa, containing a little pasty material. The entire intestinal mucosa shows very slight congestion, and it has been shown clearly that the poison concerned causes splanchnic dilatation and even paralysis. The mucosa of the loop is often slightly injected and Hartwell states that this indicates injury to the mucosa; however, the microscope shows a normal mucosa except for a slight widening of the capillaries in the villi.

We have held that the poison is elaborated by the mucosa, for when the mucosa is destroyed no poison is formed. It is conceivable that the mucosa can be disturbed so as to functionate abnormally, without any gross evidence of disturbance, and we may assume a perturbed physiological balance of the mucosa by which abnormal products are formed and secreted into the blood stream. We feel that the experiments here published give adequate basis for the belief that intoxication is the essential factor in intestinal obstruction or in the closed duodenal loop complex, and that the intoxica-

tion in the two conditions is similar if not identical, and the perverted activity of the mucosa, not an anatomical injury, is responsible for the formation of the poison.

It is clear that the cells and organ extracts of immunized animals can destroy the duodenal loop poison which is so resistant to simple digestion. The destruction *in vitro* during autolysis is quite rapid and the resultant material is non-toxic. The possibility that this immune tissue juice might be potent when given intravenously at once suggests itself. It is of course possible that the immune organ extract may destroy the poison *in vivo* and help to tide the animal over a period of acute intoxication. This point is being investigated at present.

#### CONCLUSIONS.

Dogs may be immunized against lethal doses of the duodenal loop poison by means of small doses of the loop fluid from dog or cat and by material obtained from human cases of intestinal obstruction. The immunity is transient and may disappear within a few weeks.

Dogs immunized by repeated doses of loop fluid show a definite resistance against the intoxication of a closed duodenal loop and may survive twice the usual period.

A dog that recovers from simple intestinal obstruction may possess a strong resistance to the intoxication of a closed duodenal loop, thus indicating a similar type of intoxication in the two conditions.

The sera of immune dogs are inactive when incubated with duodenal loop fluid.

The organ extracts and emulsions (liver, spleen, lung) of immune dogs rapidly destroy the loop poison during incubation *in vitro*.

This destructive property is possessed by a clear filtrate of the digested immune organs, excluding adsorption, and is lost after long periods of incubation (twelve weeks).

We are investigating the action of this immune organ extract to determine whether it can destroy the closed-loop poison *in vivo* and perhaps be of value in treatment.

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## INTESTINAL OBSTRUCTION.

### IV. THE MECHANISM OF ABSORPTION FROM THE MUCOSA OF CLOSED DUODENAL LOOPS.\*

By G. H. WHIPPLE, M.D., H. B. STONE, M.D., AND B. M. BERNHEIM, M.D.

(*From the Hunterian Laboratory of Experimental Pathology, Johns Hopkins Medical School, Baltimore.*)

In an earlier paper (1) we have shown that the poison of closed duodenal loops, which is responsible for the intoxication in that condition, is not absorbed by the mucosa of the unobstructed gut. We also tested the absorption of the same toxic fluid when introduced into recently isolated duodenal loops, and found that the dogs whose closed loops contain a double lethal dose of duodenal loop poison show no more intoxication than control dogs with empty closed loops, and die in about the same period, from which it is concluded that absorption from the lumen of the closed loop may be a secondary factor of intoxication and the important fraction is absorbed from the mucosa and not from the lumen of the loop. The observation of drained-loop dogs showing fatal intoxication confirms the conclusion that the greater part of the poison is absorbed from the mucosa itself, regardless of the amount of toxic material present in the lumen.

Similar experiments made by Davis (2) in this laboratory show that a closed duodenal loop is just as toxic when left washed and empty at operation or distended with a lethal dose of duodenal loop fluid from another animal. Davis also showed that repeated washing out of the stomach and duodenum, after ligating the bile and pancreatic ducts and establishing a low duodenal fistula, yields a certain amount of the toxic substance, a fact which may be interpreted to mean that the substance is excreted normally by the duodenal mucosa; and yet such a statement may exceed the facts. It

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has been shown by the writers that the toxic body is not present in demonstrable amounts in normal digested mucosa, but this does not preclude the possibility of its normal excretion in small amounts by the duodenum. It may be objected that the animal in the above experiment is not normal in that the bile and pancreatic juice are absent, the mucosa is constantly washed by water, and the animal is suffering from a certain amount of shock following extensive operative manipulations. The weight of evidence, however, seems to favor the possibility that the toxic substance may be excreted in small amounts by the normal mucous membrane of the small intestine. The formation of this toxic substance under abnormal conditions is rapid and abundant, as it can be demonstrated easily in the small amount of mucosa found in a closed or drained duodenal loop.

The essential point is, after all, the secretion of the toxic substance into the blood, which does not happen normally, but does occur in closed or drained duodenal loops and in intestinal obstruction. The important questions then are: What causes this changed function of the mucosa? Why does it form this poison and allow its passage into the blood? How does the mucosa readjust its equilibrium, and what may bring this about?

#### EXPERIMENTAL OBSERVATIONS.

##### CLOSED DUODENAL LOOP FILLED WITH TOXIC LOOP FLUID AT OPERATION. KILLED AFTER FOUR DAYS.

Dog O-100.—Young adult, male; weight 19 pounds.

July 16, 2 P. M. Ether anesthesia and operation. Closed duodenal loop with gastro-enterostomy made as usual. At the end of the operation the closed duodenal loop was filled with duodenal loop fluid, 50 c.c., obtained from dog O-86. This fluid had been standardized and 50 c.c. was fatal to a normal dog weighing 17½ pounds. 5 P. M. Dog recovering from operation; walks about and seems normal. 8 P. M. Some vomiting and diarrhea.

July 17. Dog appears like the usual closed-loop dog, and remains curled up quietly. Temperature 38.6° C. Weight 18½ pounds. 5 P. M. Dog appears better. Temperature 38.6° C.

July 18, 10 A. M. Dog appears fairly well. Pulse is fair. Temperature 38.5° C. Weight 17½ pounds.

July 19, 9 A. M. Condition remains the same. Pulse rather weak. Temperature 38.4° C. Weight 17¼ pounds.

July 20, 11 A. M. Temperature 38.4° C. Weight 16¾ pounds. Pulse is fair and dog does not seem gravely intoxicated. Etherized and killed.

*Autopsy*.—Performed at once. Thorax, heart, and lungs normal. The peritoneum shows beginning purulent peritonitis with about 5 c.c. of thick, fibrinous-purulent exudate in each flank. The surface is moist, injected, and shows fine grains of fibrin. The loop is distended tightly. The omentum is adherent about it. There are purplish patches showing through the wall. The ends are tightly closed and it contains 80 c.c. of thin slaty fluid having a very offensive odor. The mucosa of the loop is swollen and pinkish. There are purplish areas of inflammation with submucous hemorrhage and some superficial ulcers with obvious necrosis of the mucosa. Intestines and abdominal viscera are negative except for congestion. Liver shows cloudy swelling.

**CLOSED LOOP DISTENDED WITH TOXIC FLUID AT OPERATION. KILLED AFTER FIVE DAYS.**

*Dog O-111*.—Strong, white bulldog, male; weight 32 pounds.

July 24, 3 p. m. Ether anesthesia and operation. Closed duodenal loop with gastro-enterostomy was done as usual. At the end of the operation the closed duodenal loop was filled with 100 c.c. of duodenal loop fluid. The loop in this instance was somewhat longer than normally, but this amount of fluid distended it tightly. This fluid had undergone autolysis for weeks and filtered readily. 50 c.c. of the fluid was a lethal dose for a normal dog 20 pounds in weight.

July 25, 10 a. m. Temperature 39.1° C. Weight 31½ pounds. Dog appears intoxicated to about the usual degree noted in control dogs with simple closed loops.

July 26, 10 a. m. Temperature 39.2° C. Weight 29½ pounds. Dog in fair condition and looks exactly like control dog. There is no evidence of increased intoxication from the fluid introduced into the loop.

July 27, 11 a. m. Dog has been vomiting a good deal. Temperature 38.8° C. Weight 28 pounds.

July 28, 10 a. m. Condition about the same. Temperature 39.2° C. Weight 27½ pounds.

July 29, 10 a. m. Dog looks very weak and will probably succumb during the day. Temperature 39.2° C. Weight 26½ pounds. Etherized and killed.

*Autopsy*.—Thorax, lungs, heart, and kidneys are normal. Liver is pale and shows evidence of fatty degeneration. The loop contains a good deal of fluid and is dilated with thickened walls. The ligatures at both ends have cut nearly through the wall, allowing the escape of fluid and entrance of a small amount of bile-stained material from the stomach. The mucosa is intact everywhere except at the site of ligation. There is a slight congestion of the mucosa of the loop and of the jejunum. It is possible that with the cutting through of these ligatures and beginning of the flow of normal secretion through the loop the intoxication in this animal would be overcome and recovery would take place, but it is obvious that the introduction of a large amount of duodenal loop fluid did not intensify the intoxication noted in the first few days when the loop was closed tightly.

**CLOSED DUODENAL LOOP DISTENDED WITH TOXIC FLUID. RECOVERY.**

*Dog O-112*.—Strong bulldog, female; weight 24 pounds.

July 24, 4 p. m. Ether anesthesia and operation. Closed duodenal loop with gastro-enterostomy formed as usual. At the end of the operation the closed loop

was distended with 75 c.c. of duodenal loop fluid obtained from dog O-95. This fluid had undergone autolysis for three days, followed by filtration but not heating. 60 c.c. had poisoned rapidly a normal dog weighing 13 pounds, causing death in two hours.

July 25, 10 A. M. Temperature 38.9° C. Weight 22½ pounds. Pulse is fair and dog curled up quietly.

July 26, 11 A. M. Condition about the same. Temperature 37.8° C. Weight 21½ pounds.

July 27, 11 A. M. Dog looks much better, and is rather active. Temperature 38.1° C. Weight 21¾ pounds.

July 28, 10 A. M. Dog is fairly well. Pulse is of good tension. Temperature 38.8° C. Weight 21¾ pounds.

July 29, 10 A. M. Dog is rather sick, but not much shocked. Temperature 38.9° C. Weight 21½ pounds.

July 30, 10 A. M. Dog is much better. Temperature 38.3° C. Weight 23 pounds. Dog does not vomit and is eating food. It is evident that the ligatures have cut through and the intoxication is diminishing. Condition improved until Aug. 5, when the dog is etherized and killed. Weight 23½ pounds.

*Autopsy.*—Thoracic and abdominal organs normal. The loop shows both ligatures cut through, lying embedded in the mucosa on one side of the lumen. The lumen of the lower end is dilated to almost normal, whereas the upper end still shows some constriction. The wall of the loop is thickened and is somewhat dilated. The mucosa everywhere except at the site of ligation is intact and normal.

The three preceding experiments (dogs O-100, O-111, and O-112) indicate that the presence of large amounts of toxic, standardized duodenal loop fluid in a recently established closed loop will not modify the picture of intoxication. The control dog with an empty closed loop appears to be just as much intoxicated during the first forty-eight hours following the operation as the dogs whose closed loop is distended to its maximum capacity with old or fresh loop fluid.

The distended loops may show an early cutting through of the ligatures, with return to normal. We believe that pressure and distension are important factors in this result. A similar reaction is seen in the common bile duct when a ligature producing icterus cuts through in about five days and allows the free flow of bile, the ligature lying in the side of the duct wall. In the empty closed loop the fluid accumulates slowly and may be considerable after twenty-four to forty-eight hours, whereupon there will be violent peristalsis and a tendency for the ligatures at the ends of the loop to cut through the wall. Perforation or fatal intoxication almost always

supervene before this has taken place. But with a distended loop at the start, there will be violent peristalsis with increased tension on the end ligatures. This causes more or less pressure necrosis in the tissues below the ligatures, and the ligatures slowly cut and press into the tissue while the wall adheres behind the ligature and obliterates its track. Finally the ligature cuts through the soft mucosa and lies in the lumen adherent to a segment of the mucosa which is not cut through. The linear cut or ulcer of the mucosa heals over rapidly and the gut slowly comes back to its normal diameter and thickness. Shortly after a free flow through the loop is established the toxic symptoms vanish and the dog begins to eat and put on weight.

DRAINED DUODENAL LOOP. DEATH IN FIVE DAYS.

*Dog O-21.*—Black mongrel, male; weight  $23\frac{1}{2}$  pounds.

Jan. 8. Ether anesthesia and operation. Drained loop made as usual with a large drainage tube sewed into one end. Gastro-enterostomy.

Jan. 9. Good drainage from tube and loop washed out with water. Dog is in good condition and eats.

Jan. 10. Dog is active. Drainage has taken place and loop is washed out again with water.

Jan. 11. Dog seems well. Drainage tube has come out of the loop, but the loop is washed as usual.

Jan. 12. Evidence of distemper. The skin on the abdomen shows digestion and bile staining.

Jan. 13. Found dead.

*Autopsy.*—The ligature below the pancreatic duct had cut through, allowing bile and pancreatic juice in small amounts to escape into the loop. The other ligature is tight. The loop contains 1 to 2 c.c. of thick buttery material. Its mucosa is reddened, but everywhere normal except for congestion. The small intestine shows a diffuse injection with a red velvety mucosa and contains abundant fluid feces. The duodenum above the ligature shows a congested, reddish purple, velvety mucosa. The gastro-enterostomy is normal. Stomach shows congestion in the cardiac portion. The peritoneum is everywhere clean, but the mesenteric vessels are injected and widely distended. Spleen and liver are definitely engorged and tense. Thorax, heart, and lungs are normal. There is evidence in this case that the characteristic toxic agent was present, causing fatal intoxication associated with a great deal of splanchnic congestion. The escape of pancreatic juice and bile in small amounts did not modify the reaction in the loop.

DRAINED DUODENAL LOOP. DEATH IN SIX DAYS.

*Dog O-2.*—Weight  $17\frac{3}{4}$  pounds.

Oct. 16. Ether anesthesia, operation, and production of a drained duodenal loop, extending externally through the abdominal wound. Gastro-enterostomy.

Oct. 22. Loop had been rinsed out with water daily by inserting a catheter and using a liter or more of fluid. 9 A. M. Dog found dead.

*Autopsy.*—The peritoneal cavity is clean. The duodenal loop is normal and the lower end firmly fixed by granulation in the abdominal wound. The loop is collapsed and contains about 2 c.c. of pasty material. The mucosa close to the opening exteriorly shows a small ulcer involving the mucosa, measuring only 6 mm. in length. Elsewhere the mucosa is normal and intact, having a pale, pinkish mottled color. The other abdominal viscera are normal. The heart contains blood clots. The lungs show a few small purplish patches of consolidation not over 0.5 cm. in diameter.

DRAINED DUODENAL LOOP. DEATH IN TWENTY-FOUR HOURS.

*Dog O-24.*—Strong mongrel, male; weight 20 pounds.

Jan. 15, 3 P. M. Ether anesthesia and operation as usual. Drained duodenal loop of the usual length isolated, but both ends drained through the abdominal wound and drainage tubes sewed into the open ends. Gastro-enterostomy.

Jan. 16, 3 P. M. Dog appears very much intoxicated. Temperature is sub-normal. Loop washed out thoroughly with warm water. 4 P. M. Death.

*Autopsy.*—Performed at once. The peritoneum is clean. Thorax is normal. The blood is fluid and clotted slowly only when in contact with the body tissues, indicating the presence of antithrombin. The spleen and liver are moderately congested. The jejunum shows a mottled mucosa, reddish areas alternating with paler pink areas. The loop shows a good deal of edema and hemorrhage in the distal portions which were sewed into the abdominal wall. This was probably due to constriction and interference with the blood supply. Elsewhere the loop mucosa shows a moderate grade of congestion and is intact. It is possible that a part of this acute intoxication was due to the obvious interference with the blood supply to the ends of the drained loop.

DRAINED DUODENAL LOOP. DEATH IN THREE DAYS.

*Dog O-36.*—Large mongrel, male; weight 30½ pounds.

Feb. 5, 4 P. M. Ether anesthesia and operation. Drained loop made as usual and gastro-enterostomy established. Tubes sewed in both ends. One was brought out through a puncture wound in the right rectus, and the other through the abdominal wound.

Feb. 6, 10 A. M. Dog shows considerable drainage from the duodenal loop which is washed out thoroughly with water and a very dilute solution of permanganate. Temperature 37.7° C. 4 P. M. Dog appears intoxicated. Temperature 38.9° C.

Feb. 7, 10 A. M. Temperature 38.5° C. Dog looks better and loop is washed out thoroughly with a large amount of dilute permanganate solution. 2 P. M. Dog appears gravely intoxicated.

Feb. 8, 12 M. Much vomiting. Death.

*Autopsy.*—Performed at once. The peritoneum is clean. The stomach is full of bile-stained fluid similar to that found in intestinal obstruction. The intestine everywhere is full of a similar fluid. The mucosa of the small intestine has a patchy, injected appearance. Gastro-enterostomy is clean. Drainage tube ex-

tended well into the loop, and the mucosa in contact with the tubes is red and swollen, and there is evidence of some inflammation. There is some mucus and fluid accumulated in the dependent portion of the loop. Between the ligature just below the pancreatic duct and the upper drainage tube is a portion of intestine about two inches in length which was not drained and in which material had accumulated. Other organs are negative except for a few very small patches of hemorrhagic bronchopneumonia in one lung.

The loop showed incomplete drainage in spite of large amounts of wash fluid (permanganate), because a small bit of intestine between the upper tube and the ligature below the pancreatic duct did not open freely into the loop, the drainage tube being too large and long. The accumulation of the characteristic fluid in this small portion of the duodenum may have hastened the fatal intoxication which was acute and characteristic.

The group of dogs (O-21, O-2, O-24, and O-36) with drained and washed loops shows that intoxication may develop under such conditions, and the only possible source for the poison is the mucosa. Moreover, the toxin can be demonstrated in the mucosa of the loop and in the loop washings. In an earlier publication (3) similar cases were cited and we have now a series of experiments that prove this point beyond doubt. It will be recalled that under favorable conditions a dog with a drained loop may live in good health for many weeks, and it may now be assumed that such a dog has developed a strong immunity that protects it against the intoxication. An experiment cited below gives evidence of an immunity demonstrable in the mucosa of a drained-loop dog. The immune mucosa can destroy a lethal dose of duodenal loop fluid after incubation for a few days, while normal mucosa has no such activity.

Conditions do not seem to be as abnormal in a drained loop as in a closed loop where we may have accumulation of much toxic fluid under tension with enormous multiplication of bacteria and often some injury and ulceration of the loop wall. The drained loop is rinsed out carefully each day with much fluid, and little or no material accumulates. Yet the intoxication develops and is usually fatal in the majority of cases. We can eliminate great numbers of bacteria by the repeated washings, although, of course, bacteria are still present but probably in less numbers than in the normal gut. It is clear that the bacterial flora will be much modified in a drained loop even if not greatly lessened in amount. The normal flow of the products of digestion and excretion is obliterated and

we are inclined toward the view that it is this essential deviation from normal which causes the perverted activity of the mucosa.

**DUODENAL LOOP DRAINING INTO THE JEJUNUM. INTOXICATION AND IMMUNITY.**

*Dog O-41.*—Strong bulldog, male; weight 25 pounds.

Feb. 14, 4 p. m. Ether anesthesia and operation. Gastro-enterostomy as usual. The duodenum just below the pancreatic duct is cut across and ligated with inversion of ends and closure. This partially closed duodenal loop can drain freely into the jejunum.

Feb. 15, 10 a. m. Dog looks well. 4 p. m. Dog appears toxic, but there is no vomiting.

Feb. 16-19. Dog appears well.

Mar. 1. Dog is losing weight but appetite is good; weight 21 pounds.

Mar. 7. Animal continues to lose weight. Temperature 38.9° C. Weight 18 pounds. There is some vomiting. 4 p. m. Dog killed.

*Autopsy.*—Beginning peritonitis with abscesses forming about the duodenal section. Thorax, heart, and lungs normal. Spleen and liver swollen and grayish. Duodenal loop is empty except for a little mucus. Other organs are relatively negative. The mucosa from the duodenum, jejunum, and ileum are washed, scraped off carefully, and combined with the duodenal loop fluid (dog O-33), 30 c.c. in amount. The fluid had been standardized and shown to be toxic, 25 c.c. poisoning fatally in four hours a normal dog (No. O-46), weighing 16 pounds.

The mixture of mucosa (dog O-41) and duodenal loop fluid (dog O-33) was allowed to undergo autolysis at 38° C. for eight days, and was then heated at 60° C. for one hour, centrifuged, and filtered. This gave a clear, odorless broth, 115 c.c. in amount, which was injected intravenously into a normal dog (No. O-51), weighing 15½ pounds. It caused an initial drop in blood pressure followed by a rise (kymograph) with return to normal in thirty minutes. After six hours the dog appeared fairly well. Pulse good. There has been no vomiting and only one soft stool. The next day the dog is normal in every way. The experiment indicates that this loop dog (No. O-41) had been immunized by the presence of his partially drained loop, causing more or less intoxication. The immune mucosa *in vitro* completely destroyed with considerable rapidity a standard toxic dose of duodenal loop fluid.

**DUODENAL LOOP DRAINING INTO THE JEJUNUM. DEATH IN FOUR DAYS.**

*Dog O-19.*—Active young female; weight 17 pounds.

Dec. 14, 3 p. m. Ether anesthesia and operation. Gastro-enterostomy was done at the usual place and a single ligature placed at the site of the usual upper ligature of the closed duodenal loop. The lower ligature was not placed, so that a part of the duodenum is isolated which can drain freely into the jejunum. The gastric contents, bile, and pancreatic juice escape through the gastro-enterostomy.

Dec. 15. Dog appears normal. Temperature 38.6° C.

Dec. 16. Temperature 37.9° C. Dog seems well.

Dec. 17. Temperature 38° C. Dog has lost weight. Weight 15 pounds. At this time the dog had a violent convulsion.

Dec. 18. Temperature 36.6° C. Dog appears sick. 3 p. m. Temperature 32.4° C. Animal desperately ill and died shortly after this observation.

*Autopsy.*—The peritoneum is normal. The partially closed loop contains about 10 c.c. of bile-stained, alkaline fluid. Its mucosa is normal, except for a slight congestion which is present as well in the jejunum. There is free communication between the duodenum and the upper portion of the jejunum. The small intestine shows a mottled, pink and red mucosa and contains a large amount of fluid fecal material. Stomach contains bile-stained fluid with alkaline reaction. Gastro-enterostomy is perfect. Liver, spleen, and kidneys show some congestion. Heart contains fluid blood. The lungs show small patches of hemorrhagic bronchopneumonia in the lower lobe.

DUODENAL LOOP DRAINING INTO THE JEJUNUM. INTOXICATION.

Dog O-20.—Mongrel bulldog, male; weight 22½ pounds.

Jan. 3, 3 p. m. Dog is in poor condition. Ether anesthesia and operation. A partially isolated duodenal loop, similar to that described in the preceding experiment, was made with gastro-enterostomy in the usual location.

A single ligature was placed just below the pancreatic duct, completely occluding the lumen of the duodenum.

Jan. 4, 4 p. m. Temperature 38.1° C. Dog is sick and has diarrhea.

Jan. 5, 10 a. m. Temperature 38.7° C. Diarrhea continues.

Jan. 6, 10 a. m. Temperature 38° C. Diarrhea still more marked. Dog is very quiet and refuses food.

Jan. 7. Found dead.

*Autopsy.*—General peritonitis with a good deal of purulent exudate due to leakage at the site of gastro-enterostomy. This is a recent peritonitis, and the symptoms of intoxication which preceded its development associated with diarrhea, etc., were probably due to the intoxication from the partially closed duodenal loop.

This group of experiments (dogs O-41, O-19, and O-20) brings out two points. Ligation or section across the duodenum below the pancreatic duct together with a gastro-enterostomy just beyond the duodenojejunal junction produces a condition which we may call a partially or half closed loop. This half closed loop drains freely into the upper jejunum, and can be washed incompletely by a back flow of fluids, passing from the stomach through the gastro-enterostomy. The presence of such a partially isolated duodenal loop may be associated with intoxication, more or less severe, which will bring about an immunity reaction in the body cells. The intestinal mucosa from such a dog has the characteristic property of immune tissue; it can destroy with some rapidity the duodenal loop fluid *in vitro* and render the mixture harmless when given intravenously to a normal dog. The normal mucosa does not possess this activity.

## CLOSED DUODENAL LOOP PLUS BILE. DEATH ON SECOND DAY.

*Dog O-10.*—Fox-terrier, male; weight 19 pounds.

Nov. 6, 3 p. m. Ether anesthesia and operation. The pancreatic duct is isolated by ligature and cut. The lower ligature of the closed loop is placed in the usual situation. The upper ligature is placed between the bile duct and the pylorus. The common bile duct was left open and drained freely into the closed loop.

Nov. 7, 12 m. Dog appears sick. Temperature 36.9° C.

Nov. 8, 9 a. m. Dog found dead.

*Autopsy.*—Performed at once. Numerous fat necroses throughout the peritoneal cavity, indicating the escape of pancreatic juice at the site of operation. The duodenal loop had ruptured with the escape of bile-stained fluid and obvious peritonitis. The loop shows necrosis with ulceration of the mucosa and submucous hemorrhage and the mucosa is very soft and easily separated. The fluid is slimy, thick, and deeply bile-stained. The lungs show some edema and areas of purplish pneumonia. Other viscera are normal except for engorgement.

## CLOSED DUODENAL LOOP PLUS BILE. DEATH IN TWENTY-FOUR HOURS.

*Dog O-12.*—Mongrel, female; weight 21½ pounds.

Nov. 21, 4 p. m. Ether anesthesia and operation. The isolated duodenal loop included about six inches of jejunum, being somewhat longer than usual. The upper ligature is placed between the bile papilla and the pylorus. Gastro-enterostomy as usual. The pancreatic duct is ligated and cut.

Nov. 22, 9 a. m. Temperature 38.9° C. 1 p. m. Death.

*Autopsy.*—Performed shortly after death. The peritoneum contains a few cubic centimeters of blood-stained fluid with delicate grains of fibrin over the intestinal coils in the region of the liver. The pancreas shows a few fat necroses around the head of the organ. The loop is greatly dilated with fluid. Its wall is thin and shows subserous hemorrhages. The mucosa is intact, but obviously inflamed and swollen and scrapes off easily. The liver and spleen show a good deal of congestion. The small intestine shows a reddish congested mucosa. The other organs are negative. The duodenal loop fluid in this case was tested on a normal dog (No. O-28), and 70 c.c. given intravenously caused rapid and fatal intoxication, with death in two and one half hours. Weight 11½ pounds.

## CLOSED DUODENAL LOOP PLUS PANCREATIC JUICE. DEATH ON FOURTH DAY.

*Dog O-6.*—Black mongrel, male; weight 19 pounds.

Oct. 30, 3 p. m. Ether anesthesia and operation. Duodenal loop made with the lower ligature in the usual situation. The upper ligature is placed above the pancreatic duct, which opens freely into the closed loop. The common bile duct is isolated between the ligatures and cut.

Oct. 31. Dog does not appear intoxicated to a grave degree and takes a little food.

Nov. 1. Dog seems fairly well. Temperature 38.1° C.

Nov. 2, 9 a. m. Found dead, but quite warm.

*Autopsy.*—The peritoneal cavity is filled with turbid, yellow, bile-stained fluid.

Thorax negative except for a few patches of bronchopneumonia. Spleen and liver slightly congested. The loop shows a rupture in the middle third where the wall shows extensive ulceration and softening. On cutting open the loop the ends are found to be tightly closed and the mucosa shows extensive ulceration and necrosis with submucous hemorrhage and a great deal of ecchymotic reaction, edema, and exudation. The small intestine shows a purple, mottled mucosa with evident engorgement of the mesenteric vessels.

**CLOSED DUODENAL LOOP PLUS PANCREATIC JUICE. DEATH ON THIRD DAY.**

*Dog O-7.*—Black and tan mongrel, male; weight 15 pounds.

Oct. 31, 4 p. m. Ether anesthesia and operation. The closed duodenal loop is made as in the preceding experiment, the lower ligature being in the usual location, but the upper ligature well above the bile and pancreatic papillæ. The common bile duct is doubly ligated and cut.

Nov. 1, 9 a. m. Dog curled up quietly. Temperature 38.9° C.

Nov. 2, 9 a. m. Death.

*Autopsy.*—Performed at once. The peritoneum contains about 100 c.c. of blood-stained, turbid fluid. The serous surfaces are specked with small hemorrhages, and the reaction is like that following the injection of duodenal fluid into the peritoneal cavity. The duodenal loop shows areas of hemorrhage, necrosis, and ulceration with perforation in the middle third. The mucosa everywhere is coated with mucus, deeply injected, and swollen, with extensive inflammatory reaction. There are deep ulcers with hemorrhages in the submucous tissue. The upper ligature is not quite tight and a little exchange of fluid may have been possible. Thorax, heart, and lungs negative. Spleen, liver, and kidneys are engorged. The jejunum shows a congested and velvety mucosa with deeper purplish patches. The lower portion of the small intestine is also congested. The picture is practically identical with that which may be found following intraperitoneal injection of heated, filtered duodenal loop fluid.

**DUODENAL LOOP PLUS THE PYLORIC HALF OF THE STOMACH. DEATH ON FOURTH DAY.**

*Dog O-15.*—Strong mongrel, male; weight 45 pounds.

Nov. 30, 2 p. m. Ether anesthesia and operation. Common bile duct and pancreatic duct ligated and cut. Stomach bisected. Gastro-enterostomy made by mistake into the pyloric half of the stomach, and the upper or cardiac portion completely isolated. The partially closed loop then contained the normal amount of duodenum plus the pyloric portion of the stomach which opened into the jejunum through a gastro-enterostomy.

Dec. 1. Condition as usual. Temperature 38.3° C.

Dec. 2, 3 p. m. Condition about the same. Temperature 38.8° C.

Dec. 3, 9 a. m. Temperature 37.5° C. There is some diarrhea and much vomiting. 12.30 p. m. Temperature 38.5° C. Dog seems very sick.

Dec. 4, 9 a. m. Found dead.

*Autopsy.*—There is some fresh peritonitis about the pyloric portion of the loop, and a few fat necroses around the head of the pancreas. The loop mucosa is intact and the duodenum contains about 100 c.c. of the usual brown soupy fluid. This material is shown to contain the usual toxic material. The lungs show a few patches of bronchopneumonia in the lower lobes.

CLOSED DUODENAL LOOP PLUS THE PYLORIC HALF OF THE STOMACH. DEATH ON  
THIRD DAY.

Dog O-17.—Large male; weight 43 pounds.

Dec. 7, 3 p. m. Ether anesthesia and operation. Stomach is bisected and gastro-enterostomy is made between the cardiac portion and the jejunum. Bile duct and pancreatic duct ligated and cut. The closed loop included the pyloric portion of the stomach with the entire duodenum and about three inches of the jejunum, with a simple ligature at the lower end of the loop.

Dec. 8, 10 a. m. Dog seems fairly well. Temperature 37.7° C.

Dec. 9, 9 a. m. Temperature 37.9° C. Dog is vomiting repeatedly.

Dec. 10, 9 a. m. Found dead, but quite warm.

*Autopsy.*—The peritoneal cavity contains blood-tinged, turbid fluid, about 150 c.c. There has been a rupture of the loop in the descending arm of the duodenum, where there are extensive areas of hemorrhage and ulceration. The pyloric portion of the stomach included in the loop is somewhat reddened but intact. The small intestine shows a congested velvety mucosa. The pancreas shows a few fat necroses around the head. The lungs are normal. The heart shows several adult specimens of *Filaria imitis*.

The preceding group of experiments shows that a closed duodenal loop is quite as toxic when the pancreatic juice or bile flows into it or when it includes the pyloric portion of the stomach. In the latter instance the dogs survived slightly longer, but this may be explained partly by the fact that the dogs in these experiments were unusually large and strong. These animals may survive a simple closed loop for a period of four or even five days.

## DISCUSSION.

It will be recalled that a closed duodenal loop is isolated between two ligatures, and the continuity of the intestinal tract is established by means of a gastro-enterostomy. The intoxication under such conditions is very acute. If the lower ligature is omitted we form a partially closed loop which drains freely into the jejunum. Under the latter conditions the intoxication is less marked but obvious; immunity may result and be clearly demonstrated. With a simple obstruction of the upper jejunum a dog may live four to eight days, which is considerably longer than in the case of a simple closed loop. The intoxication is clearly of the same type; but why does the simple obstruction cause less acute symptoms?

Does the bile or pancreatic juice have the power of neutralizing or destroying the poison? This question is answered in the negative

by the last group of experiments. Moreover, the gastric juice is unable in any way to neutralize the poison formed in the loop or to lengthen the life of the animal. The drained duodenal loops give rise to the same type of intoxication, yet the lumen here is free from any fluid toxic accumulation. There is good evidence that the poison is not absorbed by the normal mucosa. Furthermore, absorption is not appreciably more rapid from a closed loop recently filled with the toxic fluid than from a simple closed loop.

This again emphasizes the fact that the mucosa is the source of the toxic absorption rather than the material accumulated in the lumen of the gut. The bile and pancreatic and gastric juices have no power to destroy the poison or check its production in a closed loop. Removal of the fluid by vomiting is not an essential feature, as the poison will not be absorbed from the lumen.

The essential feature perhaps is the flow of intestinal contents which provides the normal environment of the cells of the mucous membrane. When this flow is completely stopped, as in a closed loop, we note the most acute type of intoxication. It is probable that the abnormal fluid accumulation and perhaps bacterial growth may stimulate the mucosa to produce greater amounts of toxin. When simple obstruction is present there is an interchange of fluids, the current in the main being the reverse of normal, resulting in accumulation of fluid in the stomach and vomiting. Intoxication develops, but it is not as intense as in a closed loop, the reason for this being perhaps that fluids are still passing over the mucosa and tend to check the accumulation of the toxic substance within the mucosa which is most evident in the simple closed loops.

Our work with the toxic substance within the closed loops has brought out several points of interest bearing on its nature. It is clear that the poison is resistant to ordinary digestion or autolysis (pancreas or intestinal mucosa), and it can be boiled over a free flame, when in a non-coagulable fluid, without destruction. When the toxic substance is digested for weeks and months with normal intestinal mucosa it may be completely or partially destroyed. For this reason the duodenal loop fluid may deteriorate slowly, as in collection of the material it is easy to include a little of the mucosa. It can be shown that the mucosa of a closed loop, where the dog was

resistant and perhaps survived four days, can destroy the loop poison much more rapidly than normal mucosa. This fact must be kept in mind when making digestion extracts of the mucosa of closed loops, as prolonged digestion is apt to destroy some or all of the contained poison.

Normal organ extracts can destroy the duodenal loop poison when incubated for many weeks and the liver extract is most active, but their activity is far inferior to the same materials derived from immune dogs. When a sublethal dose of poison is introduced intravenously in a normal animal, it is probably fixed and destroyed by the tissue cells, and the capacity of destruction is greatly increased by this procedure, resulting subsequently in more or less immunity. The cell digestion and destruction of the poison is shown crudely by the incubation of mixtures of organ juices and loop poison. This suggests some ferment as the active factor.

Another point comes out clearly in our series of experiments. There seems to be a slight but definite seasonal fluctuation in the resistance of dogs to a duodenal loop, whether closed or drained. The operations have been done by the same persons and a great variety of animals have been included in the experiments. Dogs have a slightly greater resistance in the spring and summer than in the fall and winter. We believe that food, type of animal, and operative technique can be ruled out. The only possible factor is the temperature, and we believe that this is concerned in the slightly greater resistance of the dogs during the warm season.

The intoxication is associated with much loss of body heat and lowering of temperature. When the room temperature is between 80 and 90° F. it is obvious that there will be much less loss of body heat than with a room temperature of 60 to 70° F. This point is recognized in the treatment of operative shock, but perhaps is not sufficiently emphasized in connection with the treatment of intestinal obstruction and intoxication, which, when uncomplicated, may be associated with a subnormal temperature.

#### CONCLUSIONS.

Intoxication is evident in a drained duodenal loop whether it opens externally or into the jejunum and may be associated with

more or less immunity which can be demonstrated after a period of days.

Intoxication with a closed duodenal loop is identical whether the loop is left empty at operation or filled with a lethal dose of loop fluid. This again emphasizes the fact that absorption of the poison is essentially from the mucous membrane rather than from the contents of the closed loop.

The intoxication of a closed duodenal loop is not modified by the presence of bile, pancreatic juice, or gastric secretion.

Cessation of the normal flow of intestinal fluids which bathe the mucous membrane may be essentially responsible for the perverted activity of the mucosa and secretion of a poisonous material into the blood.

Animals may be slightly more resistant to closed or drained loops during the warm months, which may be explained by the increased loss of body heat in the colder months. This indicates that cases of acute intestinal intoxication with subnormal temperature may be benefited by a generous supply of artificial heat.

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## STUDIES IN TISSUE SPECIFICITY.

### II. THE ULTIMATE FATE OF MAMMALIAN TISSUE IMPLANTED IN THE CHICK EMBRYO.\*

By JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 33 AND 34

In a previous paper I have reported the fact that various mammalian tissues grew when implanted in the developing avian embryo, whereas in the adult chicken the same tissues were unable to survive.<sup>1</sup> It was later shown that the mammalian cells could be grown for long periods in the embryonic host if removed before hatching time and replanted in a young embryo.<sup>2</sup> That foreign tissue underwent no injurious change as a result of its long sojourn in the embryo was shown by the fact that growth continued when it was returned to its native species. If grafted into the adult fowl, or even into the newly hatched chick, the cells soon died and were absorbed. The quality that makes the chicken an unsuitable host for the growth of tissue of a foreign species seemed to develop some time between the eighteenth day of incubation and the first day of extra-shell life.

With the hope of throwing some light on the factor of resistance, a study has been made of the histological manifestations. The points considered are: first, the fate of an established and proliferating graft when left in the embryo during the development of the resistance; and, second, the fate of foreign tissue introduced during and after the onset of the refractory state.

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<sup>1</sup> Murphy, J. B., *Jour. Am. Med. Assn.*, 1912, lix, 874.

<sup>2</sup> Murphy, J. B., *Jour. Exper. Med.*, 1913, xvii, 482; *Proc. N. Y. Path. Soc.*, 1913, xii, 206.

*The Fate of Tissue Implanted in the Adult of a Foreign Species.*  
—The histologic changes about grafts from a foreign species introduced into the adult chicken closely resemble those observed about grafts of a chicken sarcoma in an immune animal.<sup>3</sup> In fact the histologic process which goes on when a living tissue is grafted into an animal unsuitable for its growth seems to have the same general characters, regardless of the source of the host's resistance. This is true in the case of animals naturally immune, either individually or because of variety or species; those with so called acquired immunity, traceable to recovery from an implanted tumor; and finally, in the case of those made artificially immune.<sup>4</sup>

To summarize the histological picture briefly, one finds about the strange graft an edema and reaction to the incidental trauma, followed by an increase in the fibroblasts in the surrounding host tissue, and then a budding out of the blood vessels and an infiltration of the tissues round about with small mononuclear cells. In birds, as Loeb and Addison have shown,<sup>5</sup> the accumulation of small round cells may be so marked that a considerable nodule of lymphoid tissue is formed (figure 1). The cells of the graft die more or less quickly, and in the meantime the connective tissue becomes more abundant, surrounding and invading the tissue remains, and separating the mononuclear cells into small islands. Finally the mononuclear cells disappear and the process subsides into a scar. There is a wide variation in the rapidity and extent of the reaction. This is especially noticeable when the same tissue is introduced into different species. Rat tissue in a mouse brings about a slow reaction, some of the rat cells surviving as long as nine days; and frequent mitotic figures are seen during the first few days. When, however, the rat tissue is grafted into a more distantly related species as the chicken, the rat cells are entirely disintegrated by the third day, and there is a rapid and extensive infiltration with small mononuclear cells. Some individual variation exists, but the fact holds true in

<sup>3</sup> Rous, P., and Murphy, J. B., *Jour. Exper. Med.*, 1912, xv, 270.

<sup>4</sup> For the literature, see Da Fano, C., *Ztschr. f. Immunitätsforsch. Orig.*, 1910, v, 1.

<sup>5</sup> Loeb, L., and Addison, W. H. F., *Arch. f. Entwicklungsmech. d. Organ.*, 1911, xxxii, 44.

general that the more distantly related the species, the more quickly the cells of the one will be disintegrated when introduced into the other.

*The Fate of Foreign Tissue in the Newly Hatched Chick.*—When grafts from a foreign species are introduced into the newly born chick the series of reactions are the same as those observed in the adult. The cells survive about the same length of time, and the reaction is observed to take place in the same sequence. Perhaps on the whole the round cell infiltration is not so extensive and the connective tissue reaction is more abundant, the process subsiding more quickly than in the adult animal.

*The Fate of Established Grafts of Foreign Tissue when Left in the Embryo during the Development of the Resistant Condition.*—The possibility was early considered that the cells of a foreign species, if left in the embryo during the supposed gradual development of the resistant factors,<sup>6</sup> might undergo an adaptation for growth in the adult. Such an adaption I find to be entirely absent. The refractory condition, instead of being of a gradual development, seems quickly to attain its full power. In order to study its time of onset and histological manifestations, grafts of rat sarcoma were inoculated into a number of chick embryos, long enough in advance of the refractory period to allow them to become established. The time found most suitable for the inoculations was from the 12th to the 15th day of incubation. The inoculated embryos were killed in lots of four or five at twenty-four hour intervals, beginning with the 17th day, and the grafts and their surroundings were examined histologically.

As late as the 18th day of incubation the grafts were found to be in active growth with no evidence of a defensive reaction on the part of the embryo (figure 2). On the 19th day most of the rat cells appeared to be still in good condition. Some of the cell nuclei, however, took the basic stain more deeply than normally and fewer mitotic figures were present. At the edge of the graft the connective tissue elements had begun to increase. By the 20th day the nuclei of most of the rat cells took a deep basic stain, and among the few cells retaining their normal staining reaction, a mitotic figure

<sup>6</sup> Murphy, J. B., *loc. cit.*

was rarely found. There was some necrosis in the centre of the graft. In a few cases there was a moderate amount of round cell infiltration. Some specimens showed a local accumulation of polymorphonuclear leucocytes. The uniform and striking change was in the connective tissue elements about the graft. By the 20th day they had increased markedly, forming a capsule of which strands had begun to invade the graft. Specimens, taken on the 21st and 22d day of incubation, showed a continuation of this connective tissue invasion and replacement (figure 3). On the 21st day only a few scattered rat cells could be identified in the mass of reactive tissue, and by the 22d day all of these had disappeared. At this late period small clumps of round cells were more numerous. The only traces of the graft were occasional fragments of necrotic tissue staining pink with eosin.

*The Fate of Grafts of Foreign Tissue Implanted during and after the Onset of the Refractory Period in the Embryo.*—The foregoing findings show that the refractory period comes after the 18th day of incubation. In order to study the reaction of the embryo to grafts introduced during and after the onset of resistance bits of rat tumor were inoculated on the 17th, 18th, and 19th days and were removed at twenty-four hour intervals. Examinations of these specimens gave uniform results with those observed in the preceding experiment. Grafts inoculated on the 17th day and removed on the 19th day of incubation showed an increase in the connective tissue about the graft, with perhaps an occasional group of small round cells. There was marked degeneration in the center of the graft. The cells at its edge showed an occasional mitotic figure but the nuclei of most of the rat cells took a deep basic stain. The graft itself was unvascularized although the blood vessels had increased in number around it. Many degenerate forms of tumor cells were present. The 20th day specimens showed an extension of the same process. There were fewer tumor cells with normal staining reaction, rarely a mitotic figure, and the connective tissue capsule was markedly increased, with beginning invasion and beginning organization of the graft remains (figure 4). The process had an end result resembling that seen in the fate of the organized graft. On grafts introduced as late as the 19th day some intact rat cells may be

seen up to the 22d day, but these were embedded in a thick mass of connective tissue. The round cell infiltration was more marked in these than in the other specimens.

#### DISCUSSION.

The results demonstrate that the period at which an established graft of foreign tissue in the chick embryo is checked in its growth corresponds in time with the period at which an introduced graft will no longer take. This suggests a radical change in the embryo which makes it an unsuitable host for the growth of the tissue of a foreign species. The period at which the refractory condition asserts itself is not remarkable for a sudden development or rapid growth of any important organ. The transfer of the respiratory function from the outer membrane to the lungs occurs only after the resistance has developed to a definite degree. This period from the 18th to the 21st day of incubation does not represent a period of more active growth of the embryo as a whole than do the earlier periods. It seems improbable, therefore, that the onset of the refractory condition can be correlated with a gross change in the embryo.

#### SUMMARY.

Rat tumors and other tissues of foreign species grow actively in the chick embryo until the onset of a refractory period. Grafts of rat sarcoma established and growing actively at the onset of this period show a rapid cessation of growth between the 18th and 19th days of incubation. This is followed by a widespread degeneration of the rat cells and a marked activity of the connective tissue elements in the embryonal tissue round about. An occasional specimen may show small mononuclear infiltration or rarely accumulations of polymorphonuclear cells in the neighborhood of the strange tissue. During the 20th and 21st days the connective tissue capsule increases rapidly, invading the graft and replacing it. The foreign cells disintegrate rapidly and by the 22d day have practically all disappeared. The period at which the established graft begins to degenerate, namely the 19th to the 20th day of incubation, is the one on which grafts of foreign tissue will no

longer take when implanted in the embryo. The cells at the edge of the graft of foreign tissue survive for a time. There is a rapid formation of a connective tissue capsule and an invasion of the grafts. The foreign cells here practically all have disappeared by the 22d day, leaving a mass of connective tissue. The absence of a round cell infiltration is the most marked difference in the process in the embryo as compared with that in the adult chicken. The process about the grafts in newly hatched chicks is characterized by a more active response of the connective tissue than in the adult and a more pronounced round cell infiltration than in the embryo.

#### EXPLANATION OF PLATES.

##### PLATE 33.

FIG. 1. A graft of the Jensen rat sarcoma removed after 5 days in an adult chicken. A = the necrotic remains of the rat tissue surrounded by phagocytes; B = small mononuclear cells densely infiltrating the tissues around the graft.

FIG. 2. The edge of a rat sarcoma growing in an 18 day chick embryo, showing the absence of a defensive reaction on the part of the host's tissue.

##### PLATE 34.

FIG. 3. The remains of a graft of rat sarcoma inoculated into a chick embryo on the 15th day and removed on the 20th day of incubation. A = the sarcoma cells showing many degenerate forms; B = the dense capsule of connective tissue surrounding and replacing the graft.

FIG. 4. A graft of rat sarcoma inoculated into an embryo on the 18th day of incubation and removed on the 21st day. A = a few surviving rat cells lying in a mass of necrotic material; the nuclei of the cells take the basic stain deeply; B = capsule of young connective tissue entirely walling off and replacing the graft.

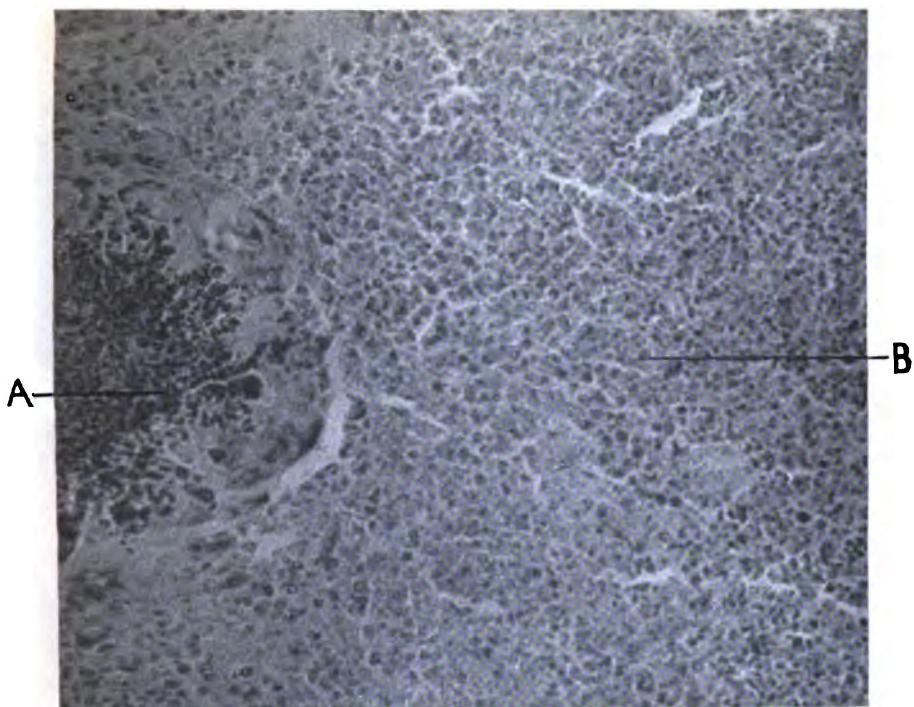


FIG. 1.

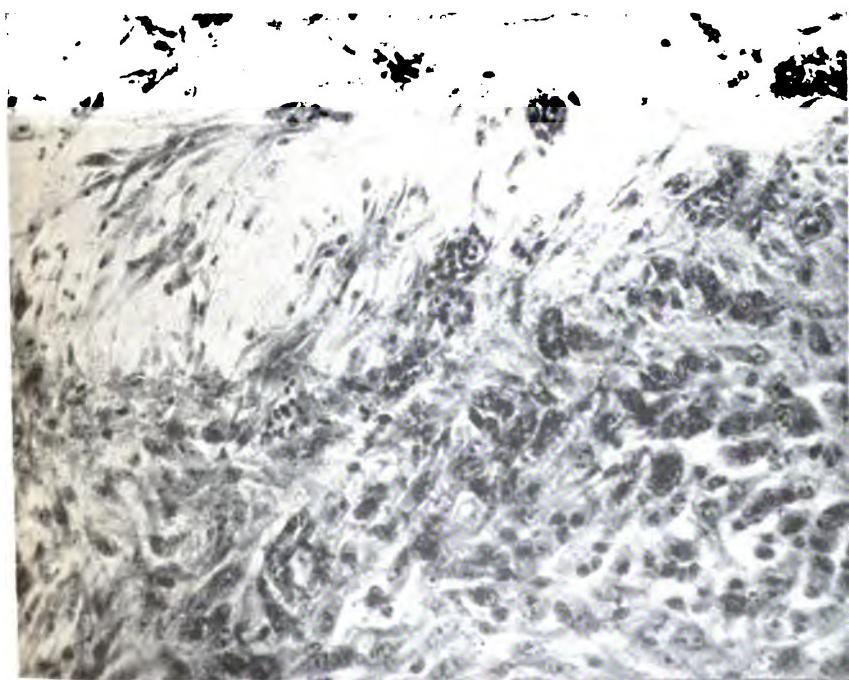
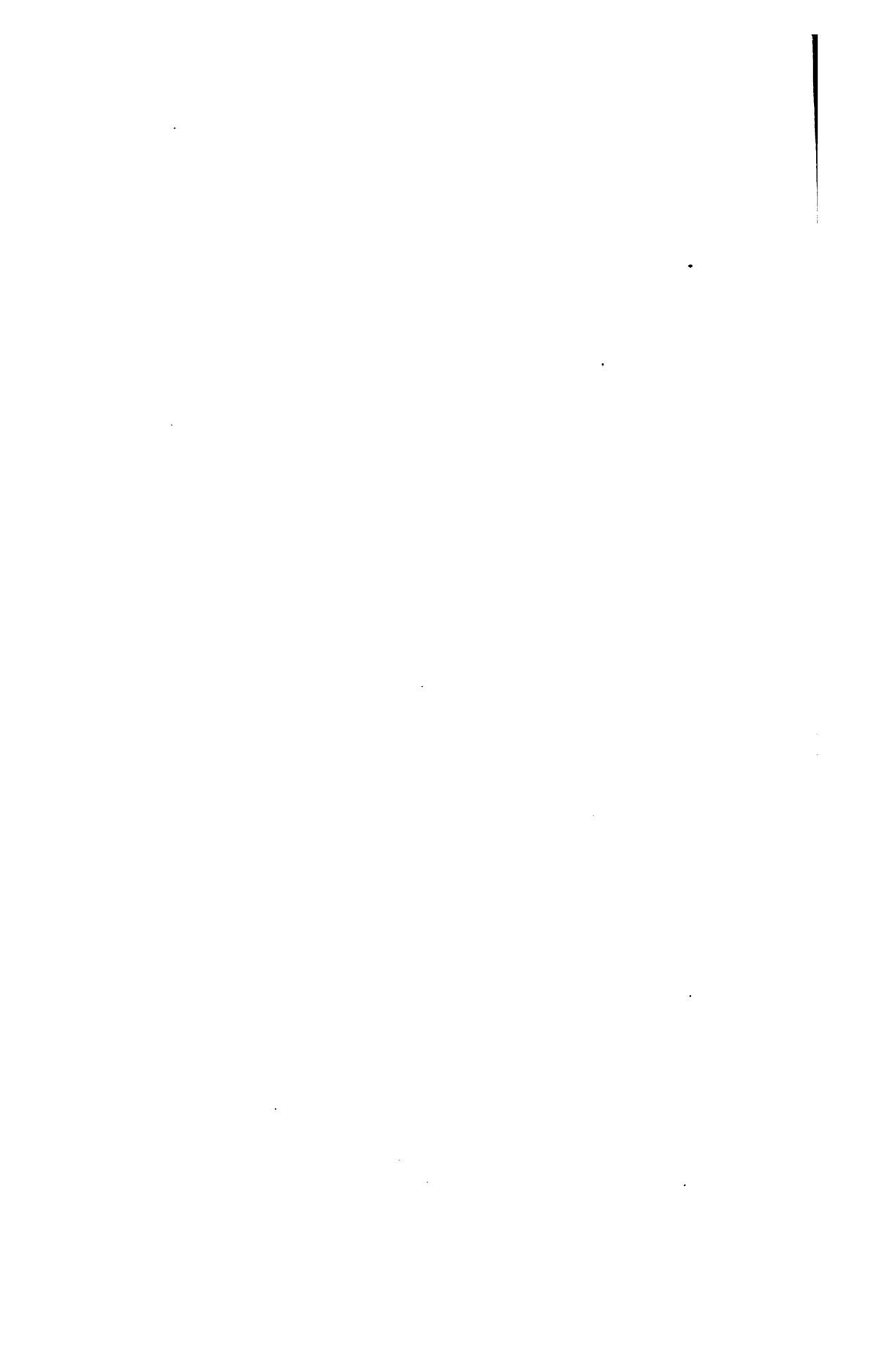


FIG. 2.

(Murphy: Studies in Tissue Specificity.)



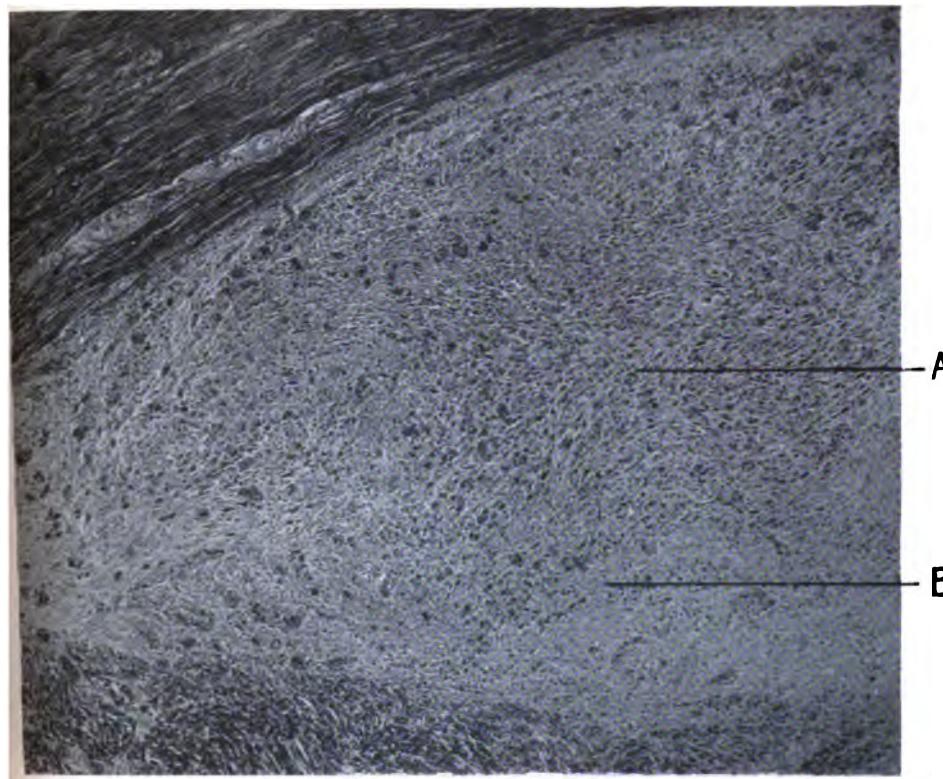


FIG. 3.

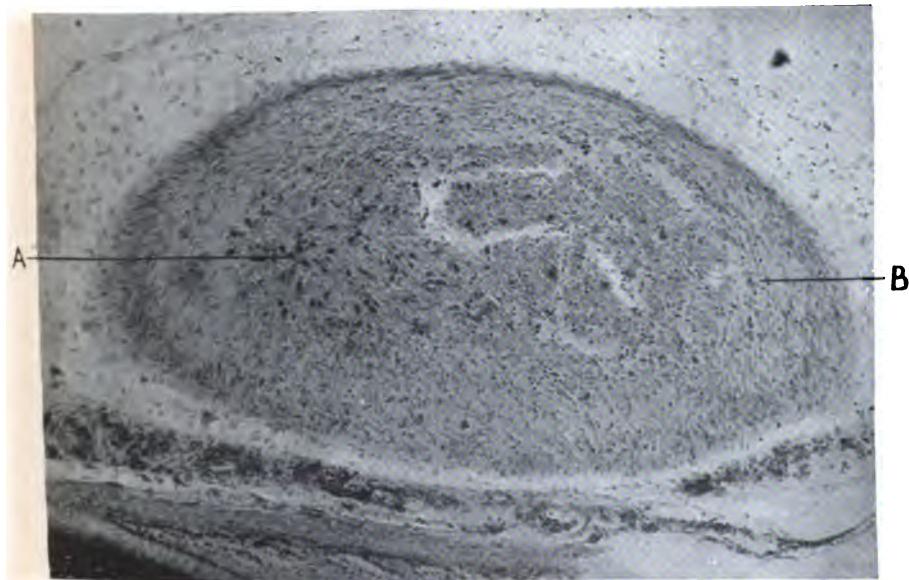
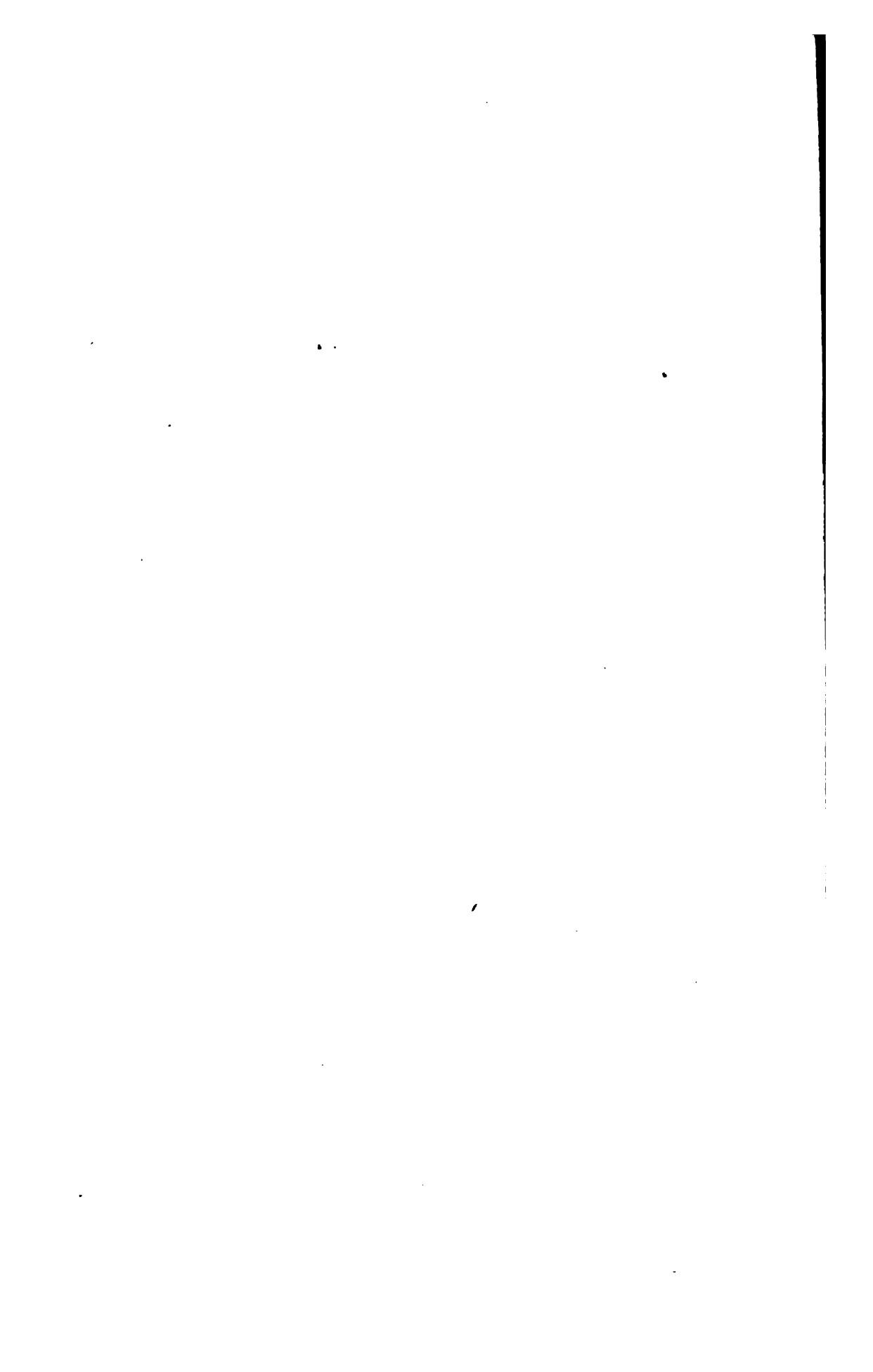


FIG. 4.

(Murphy: Studies in Tissue Specificity.)



## THE FUNCTION OF THE SPLEEN IN THE EXPERIMENTAL INFECTION OF ALBINO MICE WITH BACILLUS TUBERCULOSIS.\*

By PAUL A. LEWIS, M.D., AND ARTHUR GEORGES MARGOT.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

Experiments were begun in this laboratory three years ago to test the susceptibility of white rats and white mice to infection with *Bacillus tuberculosis*. It was expected that the animals would show a high degree of resistance to the infection, but it was hoped that they might react with such regularity as to make them available for testing various therapeutic measures. This expectation was in no way fulfilled. It developed that the animals were, on the whole, susceptible to infection, but that there was much irregularity in the reaction of individual animals.

The first experiments were made with rats. It was noted that a constant lesion in these animals was a great increase in the size of the spleen, which occurred whenever the animals survived two weeks or more. It was also noted that in several instances where the animals died in the second week after inoculation the spleen was small and extremely hemorrhagic. It seemed not impossible from a consideration of these facts that the spleen might be an important factor in the resistance of the animal to the infection, and that irregularity in the reaction of this organ might account in considerable degree for the irregularity of reaction of the animal as a whole. The same general facts were observed in a repetition of the experiments in mice.

A number of mice were splenectomized and later tested for their resistance, in comparison with intact controls. Contrary to expectation the splenectomized animals were found to have more than

\* Read before the Pathological Section of the American Association for the Study and Prevention of Tuberculosis, Washington, May, 1913. Received for publication, December 6, 1913.

normal resistance. In fact a number of animals without spleen have survived for months after inoculation with amounts quickly fatal to normal animals. In this paper we shall present the data on which the foregoing statements are based, with such observations as we have so far made that bear upon the cause of the increased resistance.

We are aware of no literature bearing on the main points at issue although the virulence of the tubercle bacillus for rats and mice, and the pathological anatomy of experimental tuberculosis in these animals, has been the subject of considerable study.<sup>1</sup>

The removal of the spleen was carried out under ether anesthesia. After etherization the mice were tied out on a frog board. The abdominal region was shaved and the spleen delivered through a suitable incision. The main blood vessels were ligated in the omentum, and the organ was then cut off. In closing the laparotomy wound the muscles and skin were separately sutured with fine silk. The animals were kept for a number of hours after operation in a warm place, free from draught. The operation was well borne; a small proportion of the animals died, either under ether or, within a few days, from hemorrhage or peritonitis. Animals surviving beyond the first week seemed to be in good health. The inoculations have never been made in less than two weeks after operation, and have usually been done in the third week.

The cultures used for inoculation were of bovine type. Culture bovine C was isolated in 1912 from a case of spontaneous bovine tuberculosis. This culture is somewhat more virulent for mice than culture R<sub>3</sub>, also of bovine type, isolated in 1911 from a human case of cervical adenitis.

The observations are presented in the following tables.

Table I illustrates the constancy and also the degree of enlargement of the spleen when rats are infected with fatal doses of tubercle bacilli.

Table II gives the results of all the inoculations made into normal mice with one, two, and five milligrams of culture bovine C. The culture has never failed to kill in these doses. The maxi-

<sup>1</sup> The literature is reviewed by Goldmann, E., *Neue Untersuchungen über die äussere und innere Sekretion*, Tübingen, 1912, 52.

TABLE I.  
*Relation of Spleen Weight to Body Weight in Rats.*

Normal rats.		Tuberculous rats. <sup>2</sup>	
Body weight.	Spleen weight.	Body weight.	Spleen weight.
123 gm.	0.26 gm.	140 gm.	1.46 gm.
135 gm.	0.43 gm.	150 gm.	1.15 gm.
130 gm.	0.37 gm.	115 gm.	1.30 gm.
129 gm.	0.71 gm.	135 gm.	2.08 gm.
150 gm.	0.42 gm.	110 gm.	2.01 gm.
125 gm.	0.51 gm.	130 gm.	2.05 gm.
		115 gm.	1.60 gm.
		120 gm.	2.20 gm.
		125 gm.	1.01 gm.
		145 gm.	0.63 gm.
<b>Average 132 gm.</b>	<b>0.45 gm.</b>	<b>128.5 gm.</b>	<b>1.57 gm.</b>
<i>Second Series. Average of Six Rats.</i>			
70.6 gm.	0.20 gm.	Average weight lost..... 8.3 gm.	

TABLE II.  
*Normal Mice. Culture Bovine C.*

Dose.	Lived.	Exudate.	Spleen.	Liver.	Heart blood.	Lung.	Kidney.
1 mg.	14 dys.	+	+	-	-	-	-
1 mg.	5 dys.	+	+	-	-	-	-
1 mg.	13 dys.	+	+	+	o	+	+
1 mg.	13 dys.	+	+	+	+	+	+
1 mg.	13 dys.	+	+	+	o	+	+
1 mg.	14 dys.	++	+	+	+	+	+
1 mg.	14 dys.	++	+	+	+	+	+
1 mg.	10 dys.	++	+	+	o	+	+
1 mg.	19 dys.	++	+	+	o	+	+
1 mg.	19 dys.	++	++	+	+	+	+
2 mg.	10 dys.	+	+	+	o	o	o
2 mg.	13 dys.	+	+	+	o	+	o
2 mg.	14 dys.	+	+	+	o	+	+
2 mg.	20 dys.	+	++	++	+	+	+
2 mg.	20 dys.	++	++	+	+	++	+
2 mg.	33* dys.	++	+++	+	+	++	+
5 mg.	13 dys.	++	+	+	o	+	+
5 mg.	13 dys.	+	+	+	+	+	+
5 mg.	14 dys.	++	+	+	o	+	+
5 mg.	14 dys.	++	+	+	+	+	+
5 mg.	14 dys.	++	+	+	+	+	+

++ = many tubercle bacilli; + = few tubercle bacilli; o = no tubercle bacilli; - = examination for tubercle bacilli not made; \* = nodules in lungs and heart muscle.

<sup>2</sup> The tuberculous animals were killed twelve days after the intraperitoneal inoculation of 10 mg. of the culture.

mum length of life was thirty-three days. At autopsy the animals frequently showed a small amount of thick yellow exudate in the omentum. The spleen was greatly enlarged. The one mouse that lived over thirty days showed numerous miliary nodules in the lungs. The nodules were softer and showed less organization than tuberculous masses of the same relative size as they are found in other species of experimental animals. The organs and frequently even the heart blood contained enormous numbers of tubercle bacilli.

TABLE III.  
*Splenectomized Mice. Culture Bovine C.*

Mouse No.	Dose.	Lived.	Perito-neal dry exudate.	Perito-neal fluid.	Liver.	Kidney.	Lung.	Heart blood.	Remarks.
1	1 mg.	5 dys.	o	+	o	o	o	o	
2	1 mg.	18 dys.	o	o	o	o	o	o	
3	1 mg.	32 dys.	o	o	o	o	o	o	
4	1 mg.	34 dys.	+	-	+	o	-	o	
5	1 mg.	37 dys.	o	o	o	o	o	o	
6	1 mg.								Still living.
7	1 mg.								Still living.
8	1 mg.								Still living.
9	5 mg.	2 dys.	o	+	o	o	o	o	Nodules in lungs and heart.
10	5 mg.	29 dys.	+	-	+	o	+	+	
11	5 mg.	32 dys.	+	-	+	o	+	o	Nodules in lungs.
12	5 mg.	32 dys.	+	-	+	+	+	o	Nodules in lungs and liver.
13	5 mg.								Still living.

+= few tubercle bacilli; o= no tubercle bacilli.

Table III gives the results of the first series of inoculations of splenectomized mice. It will be noted that two of the mice died within the first few days after inoculation. It is unlikely that the tubercle bacilli inoculated were responsible for the deaths. However, even if we include these presumably accidental losses, it is seen that the length of life in the splenectomized mice is much longer on the average than in the case of the normal mice, as shown in table II. Moreover, at the present writing (October, 1913), more than six months after inoculation, four of the animals are still alive and seem to be in perfect health. The increase of resistance, as brought out in this series, has been repeatedly demon-

strated in similar experiments or in slight modifications of them. It is of interest that these splenectomized mice, dying after the thirtieth day, show the same formation of nodules in the lungs that is found in the occasional normal mouse, surviving for an equal time. The bearing of this fact will be considered later.

It will further be noted that, as shown in tables II and III, the presence and distribution of the tubercle bacillus in the splenectomized animals that died was much more limited than in the intact animals. This suggested the possibility that in the splenectomized animals there might be a rapid destruction of tubercle bacilli which did not prevail in the intact animal. With this idea in mind we have studied the distribution of the tubercle bacillus in the first hours after intraperitoneal inoculation in splenectomized as compared with normal animals. The result of this study has been to show that in the normal animal, tubercle bacilli appear in the substance of the spleen, in the portal vein, in the liver, in the bile, and in the lumen of the small intestine within four hours after inoculation in sufficient numbers to be found readily. In splenectomized mice, on the contrary, we have never observed them outside the peritoneal cavity in less than seven days.

Within the peritoneal cavity there is no evidence of rapid lysis or increased phagocytosis. The splenectomized mice show more fluid exudate than the controls, but the significance of this fact is by no means apparent. It would seem, therefore, that the removal of the spleen has interrupted a path by which the tubercle bacillus is excreted from the normal mouse with some rapidity. These observations tend to emphasize the increased resistance brought about by the splenectomy, but in no way explain the cause of it.

In searching for an explanation of the increase of resistance, several possibilities demand consideration. In the first place it might be considered that the spleen formed a most favorable focus of infection as compared to the other organs of the body, and that with the removal of the organ much of the available food stuff is removed. Direct evidence as to the value to be assigned to the organ on the basis of these considerations is difficult to obtain.

The facts presented in regard to the limitation of the distribution of the bacilli in the splenectomized mice suggest that perhaps the

chief effect of the operation is to convert the general infection into a local one by interrupting mechanically a path of transmission. In order to test this supposition we have infected comparative series of animals in the pleural cavities. The results of this experiment as presented in table IV show that the increased resistance also prevails under these conditions. The infection tends to remain localized in the pleural cavities in this case.

TABLE IV.  
*Controls. Culture R<sub>s</sub>. Intrapleural Inoculation.*

Mouse No.	Dose.	Lived.	Pleural fluid.	Peritoneal exudate.	Spleen.	Liver.	Kidney.	Heart.	Lung.	Remarks.
1	1 mg.	24 dys.	+	+	+	+	+	o	++	Killed. Nodules in lungs.
2	1 mg.	27 dys.	++	+	+	+	+	o	++	Killed.
3	1 mg.	36 dys.	++	++	+	+	+	+	+	Pleural adhesions. Nodules in lungs.
4	1 mg.	37 dys.	++	++	+	+	+	+	+	Nodules in dia-phragm. Adhesions of pleura.
5	1 mg.	39 dys.	+++	+	++	++	++	+	++	Nodules in mediastinum.
6	1 mg.	49 dys.	++	+	++	++	++	o	++	Nodules in pleura.
7	1 mg.	49 dys.	+	+	++	++	++	o	++	Nodules in dia-phragm.
8	1 mg.	49 dys.	++	+	++	+	++	+	+	Nodules in lung and heart.
9	1 mg.	61 dys.	++	+	++	+	+	+	++	Nodules in right axillary region surrounding a blood vessel.
10	1 mg.	61 dys.	++	+	++	+	+	o	++	

*Splenectomized Mice. Culture R<sub>s</sub>. Intrapleural Inoculation.*

11	1 mg.	25 dys.	++	o		o	o	o	+	Killed.
12	1 mg.	27 dys.	+	+		o	o	o	+	
13	1 mg.	48 dys.	++	o		o	o	o	+	Killed. Few nodules in lung.
14	1 mg.	70 dys.	+	o		o	o	o	++	
15	1 mg.	70 dys.	++	o		o	o	o	++	Few nodules in lung.
16	1 mg.	125 dys.	++	o		o	+	+	++	Many nodules in lung.
17	1 mg.	125 dys.	+	o		o	o	o	++	
18	1 mg.	133 dys.	++	+		o	o	o	+	Nodules in lung.
19	1 mg.	Living								
20	1 mg.	Living								

We are, therefore, forced to the supposition that following the removal of the spleen there is a general physiological effect which

increases resistance. It has been mentioned in commenting on the results presented in table III that the mice that die after several weeks in the splenectomized series show the formation of gross nodular lesions comparable to those seen in the occasional normal mouse which survives for an unusual period. It is also notable that in the peritoneal and pleural cavities there is a plastic exudate similar to that found in the intact animals. This plastic exudate is accompanied by more fluid in the case of the splenectomized animal, whether the infection is intrapleural or intraperitoneal, but this does not furnish an obvious clue to the cause of the changed resistance.

The character of the various exudates found in the splenectomized series of animals seems to indicate that the removal of the organ does not radically change what we may call the capacity for exudation possessed by the body. The infection in the animals without spleen tends to remain localized. Experiments in progress may, we hope, show on what this limitation of distribution depends.

One control experiment should be mentioned. It is a well known fact in clinical medicine that tuberculous peritonitis is often greatly benefited and sometimes apparently cured by laparotomy without continuous drainage. As a comparable operation we have therefore removed one kidney from a number of mice by transperitoneal operation. When subsequently infected with tubercle bacilli the nephrectomized mice have reacted as do normal animals.

The change of reaction that is produced in mice by the removal of the spleen is one of the few striking instances of an increased resistance to an experimental infection with *Bacillus tuberculosis*. It would be of great importance if it should be proven that the resistance of other species of animals could be similarly altered. Experiments on guinea pigs and dogs to test this point have so far failed to bring out any change in resistance.

#### SUMMARY.

Infection of rats and mice with *Bacillus tuberculosis* (bovine type) develops a splenic tumor as a typical lesion.

Removal of the spleen from mice (albino) greatly increases their resistance to the infection. This increased resistance cannot

be explained at present. The infection in the splenectomized mice tends to remain localized as contrasted with an almost septicemic type of disease which is usual in the normal animal. The animals of each group that live more than thirty days are apt to present typical exudative lesions. The removal of the spleen does not therefore grossly change what may be called the capacity of the body for exudation.

## A CONTRIBUTION TO THE EPIDEMIOLOGY OF POLIOMYELITIS.\*

By SIMON FLEXNER, M.D., PAUL F. CLARK, PH.D., AND  
HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

As compared with the period prior to 1909, when the first experimental transmission of epidemic poliomyelitis to the lower animals was accomplished, our present knowledge of the pathology of the disease may be said to be comprehensive.<sup>1</sup> We now possess information in many ways accurate and full regarding the causative microorganism, its portal of entry into and paths of exit from the body, the period of its persistence in the tissues, the places of its location among the organs, the manner in which its presence brings about the characteristic lesions and symptoms of the infection, and certain important immunity reactions which it displays. The data upon which this knowledge is based is being extended by experiment; but up to the present the experimental studies have not yielded results that illuminate particularly the epidemiology of the affection. The observations recorded in this paper are believed to bear upon that aspect of the disease.

### PATHOGENICITY OF THE VIRUS.

Hitherto by the virus of poliomyelitis there has been understood an emulsion or filtrate prepared from infected tissues, originally of human origin, capable of transmitting poliomyelitis to monkeys. As long as the virus had not been cultivated artificially or rendered visible no other criteria than successful inoculation served to identify it. In spite of the cultivation of the organism outside the body the above definition holds in practice, for the reason that cul-

\* Received for publication, December 15, 1913.

<sup>1</sup> Landsteiner, K., and Popper, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, ii, 377. Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1639.

tivation is still difficult and in inexperienced hands uncertain, and because also less account can be taken of the artificially cultivated virus in considering pathogenicity as its virulence is readily lost.<sup>2</sup>

Somewhat conflicting results have been recorded as to the power of infected human tissues (usually parts of the central nervous organs) to convey paralysis to monkeys. The number of successful implantations has varied from one half or less of the specimens tested<sup>3</sup> to all those inoculated.<sup>4</sup> No conclusion regarding the virulence of the original materials can be drawn from these figures which often are based upon a few tests in which wide differences in method play a part. One exception only to this generalization may have to be made. Of all countries visited recently by epidemic poliomyelitis Sweden seems to have suffered most severely. Elsewhere than in Sweden it has been found difficult to communicate experimental poliomyelitis to monkeys with filtered washings of the nasopharyngeal mucous membranes from acute cases.<sup>5</sup> The deduction seems obvious, therefore, that the Swedish virus, as it exists in nature, is more active for monkeys than the strains occurring in other countries. And yet the conclusion cannot be accepted unreservedly since even small differences in the methods employed for preparing the washings may account for the discrepancy. Thus far in Sweden the virus, which is believed to be carried by healthy persons, has not been transmitted to monkeys so as to set up the typical experimental paralysis, in spite of many trials, while it has been inoculated successfully in the United States.<sup>6</sup> There is nothing inherently improbable in the supposition that the virus of immediate human origin in one place should be more active because better adapted for monkeys than elsewhere at the same period. But in becoming adapted to the monkey the virus undergoes profound modifications; therefore caution is needed before the view is

<sup>2</sup> Flexner, S., and Noguchi, H., *Jour. Exper. Med.*, 1913, xviii, 461.

<sup>3</sup> Landsteiner, K., in Kolle-Wassermann, *Handbuch der pathogenen Mikroorganismen*, 2d edition, Jena, 1913, viii, 427.

<sup>4</sup> Flexner, S., and Clark, P. F., *Jour. Am. Med. Assn.*, 1911, lvii, 1685.

<sup>5</sup> Kling, C., Wernstedt, W., and Petterson, A., *Ztschr. f. Immunitätsforsch. Orig.*, 1911-12, xii, 316, 657; 1912, xiv, 303.

<sup>6</sup> Flexner, S., Clark, P. F., and Fraser, F. R., *Jour. Am. Med. Assn.*, 1913, lx, 201.

adopted that such already modified strains exist in nature. And yet it appears that in Sweden and probably in still other countries the virus fluctuates considerably in its effects upon human beings, as indicated by the varying prevalence, severity, and mortality of the epidemics of poliomyelitis.

By successive passages of human strains of the virus through monkeys a high degree of virulence may be attained for this species. How great the changes are that take place can be inferred only, since in the inoculation of filtered extracts of the nervous and other organs we cannot actually measure the number of micro-organisms introduced. But where the original materials may prove infective only when in a state of an emulsion of which several cubic centimeters may be required, ultimately 0.1 to 0.001 of a cubic centimeter of Berkefeld filtrate may suffice to cause paralysis. The rise in virulence thus indicated is not determined merely by the smaller effective dose but also by the circumstance that while at the beginning of the adaptation the proportion of monkeys developing paralysis is smaller and the number of recoveries after paralysis larger than at a later period, once the adaptation has been accomplished all the animals inoculated tend to become paralyzed and to succumb to the disease.<sup>7</sup>

After it had acquired a state of high virulence the virus retained maximal activity for monkeys over a long period of time. But whether this maximum becomes a fixed quality was not known. This point is one of fundamental importance as regards, among other things, the question of the causes responsible for the rise and fall of epidemic waves of the disease in nature. We have carried a particular strain of the poliomyelic virus (M A) through a series of monkeys beginning in the autumn of 1909<sup>8</sup> and ending in the autumn of 1913. At the outset the virus conformed in activity to the description given: at first infection was irregular and recovery after paralysis not uncommon. After several passages adaptation of the virus was secured and infection followed more regularly and then constantly upon inoculation for which small doses of the filtrate

<sup>7</sup> Flexner, S., Huxley Lecture, *Lancet*, 1912, ii, 1271, and *Science*, 1912, xxxvi, 685.

<sup>8</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1639.

sufficed. Recovery at first rarely and later never occurred.<sup>9</sup> For precautionary reasons the inoculations were usually made in duplicate or multiple series. At the same time portions of the spinal cord and brain from infected animals were regularly set aside in 50 per cent. glycerin in the refrigerator, so that if an inoculated monkey succumbed to an intercurrent disease,—dysentery, pneumonia, or tuberculosis,—as occasionally happens, the strain would not be lost.

The M A strain retained maximal virulence during a period of about three years, from the winter of 1909 to the winter of 1912, when a change became apparent. The power to infect began to fluctuate, while among the paralyzed animals recovery began to take place. Once begun the infective power diminished more and more although not regularly. Even at this time several successive inoculations might be succeeded by infection and death. But gradually and somewhat irregularly the virulence deteriorated more and more until the state of the virus resembled in its infecting power that of the original human material from which it was derived. At first the filtrates and only later the emulsions acted irregularly and unreliably. The infective power was not, however, completely abolished, for paralysis could still be induced in occasional animals by means of large doses of filtrates or emulsions, but by no means certainly, irrespective of the size of the dose administered.

During the long period of maximal infectivity the virus seemed to have acquired established or fixed virulence. It now became obvious that fixation of pathogenic effect in the sense in which this term is applied to the vaccine or rabic virus did not occur. Moreover, the return of the maximal virus to a state similar to that of the original human source is apparent only at the present time since thus far no tendency to rise again has been noted in the deteriorated strain. This is true of all the collateral specimens of the M A strain carried through the several series, the deterioration not being confined to a single specimen of the strain. Whether after a resting period a second enhancement can be accomplished by a new series of passages cannot of course be predicted. But

<sup>9</sup> Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

that the deterioration began at a time considerably previous to its discovery is shown unmistakably by our attempts to secure a vigorous strain by returning to glycerinated specimens set aside during the still active period of the virus. After a varying number of passages fluctuation again set in and a similar irregularity in pathogenic effects appeared necessitating abandonment of the series. Long glycerination itself preserves the virus in the state of activity possessed at the time it was set aside. By resorting to a specimen (K) originally also adapted in 1909<sup>10</sup> and passed through an occasional monkey at long intervals, a highly virulent strain was again secured for experimental purposes. If a comparison of the two strains M A and K, adapted at about the same time, is permissible the conclusion to be drawn from their relative present states of activity is to the effect that frequent and long continued passage through monkeys finally brings about a depression of virulence, while preservation in a state of latency for a period equally great exerts no depressing action.

When the M A virus became highly potent the inoculation series were continued in two general ways. What was termed the regular series was designed merely to secure a constant passage virus. For this purpose Macacus monkeys were systematically inoculated, usually intracerebrally, with 1 or 2 c.c. of a Berkefeld filtrate prepared from a 5 per cent. suspension of the fresh medulla and spinal cord of a recently paralyzed animal. The disease thus caused ran the typical course of experimental poliomyelitis. The rule was to etherize the paralyzed animals within this series. Coincidentally with the regular series other series of inoculations were conducted in the course of the study of special aspects of the problem of poliomyelitis. The filtrate for this second group of experiments was usually given in smaller doses. The active virus caused paralysis in quantities of filtrate varying from 0.1 to 0.01 c.c. or less; hence the doses employed ranged from 0.1 to 0.5 c.c., depending on the purpose of the injection. The incubation period of the series receiving the smaller doses was somewhat greater than that of the regular series, but the final result was the same. In some instances intrasciatic<sup>11</sup> and combined intrasciatic and intraperitoneal injections<sup>12</sup> were employed. An active virus causes infection by intraneurial injection almost as constantly as by intracerebral inoculation. Falling off in virulence is expressed in (a) failure to cause paralysis, (b) mild infection followed by recovery, and (c) by atypical symptoms and clinical course, followed by either recovery or delayed paralysis and death. The typical course is characteristic. Following an

<sup>10</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1913.

<sup>11</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1913.

<sup>12</sup> Leiner, C., and von Wiesner, R., *Wien. klin. Wchnschr.*, 1909, xxii, 1698; 1910, xxiii, 91.

incubation period of from five to seven days there succeed excitement, general tremor, weakness, and then paralysis of muscles affecting first the extremities, next the trunk, and lastly the respiration. Exceptionally the muscles of respiration are affected early and convulsions and death occur before paralysis of the extremities is noted. When the course is atypical either reinoculation or microscopical examination of the spinal cord may be required to establish the infection.

The fact of the loss of power of the M A strain of the virus is brought out in table I. Other instances showing this loss have not been included. These were examples of border-line inoculation with smaller doses of virus; but they serve to exclude still further any effect of resistance as such as being a determining factor among the monkeys.

TABLE I.  
*Illustrative Examples of Weakened Virus.*

Date of inoculation.	Materials inoculated.	Clinical result.	Remarks.
Aug. 24, 1912	Emulsion of spinal cord	Typical paralysis	Death on 6th day.
Aug. 26, 1912	Filtrate of preceding emulsion	No effect	
Sept. 1, 1912	Emulsion	Atypical paralysis	Death on 8th day.
Sept. 23, 1912	Filtrate	Atypical paralysis	Recovery; immune.
Oct. 7, 1912	Emulsion	Atypical paralysis	Death on 11th day.
Dec. 9, 1912	Filtrate intrasciatic	No effect	Two animals used.
Dec. 18, 1912	Filtrate	Atypical paralysis	Recovered.
Jan. 21, 1913	Emulsion intrasciatic	No effect	Two animals used.
Feb. 8, 1913	Filtrate 0.1, 0.2, 0.3 c.c. intracerebral	Paralysis not strictly typical	Three animals used.
Apr. 17, 1913	Filtrate	Typical paralysis or no effect	Of 4 animals 2 escaped paralysis.
July 2, 1913	Emulsion	Slight ataxia; no progress	Recovery.
July 9, 1913	Emulsion	Atypical paralysis	Recovery.
July 10, 1913	Emulsion	Partial paralysis	Recovery with residual paralysis.
July 19, 1913	Filtrate	Typical paralysis; prostate	Recovery with residual paralysis.
July 21, 1913	Filtrate	Typical paralysis; prostate	Recovery with residual paralysis.
Aug. 26, 1913	Filtrate	Ataxic; partial paralysis	Recovery with residual paralysis.
Sept. 9, 1913	Emulsion	No paralysis	Four other animals inoculated with filtrate from this emulsion without effect.
Sept. 10, 1913	Filtrate	Partial paralysis; rapid partial recovery	Recovery with residual paralysis.
Oct. 23, 1913	Emulsion	Partial paralysis	Recovery with residual paralysis.

BEARING ON EPIDEMIOLOGY.

We do not possess a generally acceptable theory to account for the epidemic waves of disease. What is required is an adequate explanation of the initial rise, persistence, and the final fall of the wave as represented by the varying number of the affected. That mere presence of the microbic causes of disease does not suffice to produce epidemics has long been known. It is just the discrepancy between the occurrence of the microbic causes in sporadic cases of potentially epidemic diseases and the absence of true epidemics that has led to the formulation of the hypothesis of concomitant causes of von Pettenkofer and of Nägeli. While the one supposes a necessary ripening of the microbic agent in the earth as a prerequisite, the other invokes the coöperation of a second although unknown but subsidiary microorganism.<sup>13</sup> The subject has not been rendered essentially more comprehensible by the discovery of the healthy and chronic carriers of infectious microorganisms, or by the more ready detection of so called abortive cases of infection. Indeed, these discoveries only add to the perplexity since they prove that potentially infective microorganisms capable of starting epidemics are more frequently present in our surroundings than has hitherto been supposed.

Perhaps a factor which has not up to the present been sufficiently considered is that of variations among the microorganisms themselves that may be directly responsible for the production of epidemics. That microorganisms, along with all living things, tend to vary in their biological properties, has long been known, but it is only recently that these variations have been recognized as constituting mutations.<sup>14</sup> The variations thus far studied relate chiefly to (a) colony formation and fermentation effects, and (b) serum and drug reactions of fastness, while variations affecting the quality of virulence have been little considered in this respect.

The number of examples known in which mutation in the quality of virulence has occurred is already considerable. Thus per-

<sup>13</sup> Gotschlich, E., in Rubner, M., von Gruber, M., and Ficker, M., *Handbuch der Hygiene*, Leipzig, 1913, iii, pt. I, 206.

<sup>14</sup> Müller, R., *Ztschr. f. indukt. Abstammungs- u. Vererbungslehre*, 1912, viii, 305.

manent alterations of this character have been produced in vaccine virus and the fixed rabic virus. Not a few pathogenic bacteria may be changed profoundly in virulence by animal passages, oftenest with the effect of intensification but not infrequently with the contrary effect. Even in artificial cultures enhancement as well as diminution of virulence has been noted and particularly in the case of plague bacilli and meningococci.<sup>15</sup> The modifications in virulence appear at one time quickly or even suddenly and at another develop gradually. There is no doubt that under many natural conditions the passage of infectious microorganisms rapidly from animal to animal or person to person leads to great enhancement of virulence as, to mention one example only, in the pneumonic form of the plague. There exists experimental foundation for the belief that during the rise the microbial causes are more virulent than during the fall of epidemics. The questions to be answered are whether it is this variation, perhaps mutation, among the potential microbial causes of epidemics that is responsible for the waves mentioned, and if so what the nature of the agency is that brings about the mutational changes. It is because the data bearing on the cycle of pathogenicity of the M A virus seem to offer answers to these important questions that they have been set down here in detail.

At the outset the virus of human poliomyelitis possesses relatively weak pathogenic action for monkeys. By means of a few passages the infective power rises and soon a maximum is reached which endures for several years. Ultimately, the infective power falls off and soon becomes greatly diminished, so that finally the power is no greater than at the outset. This succession of phenomena dependent on changes in virulence finds a counterpart in the phenomena noted during the rise, persistence, and then fall of numbers of cases, among man and animals, that constitute epidemics of disease. Moreover, the fluctuations in virulence upon which the phenomena in the first instance depend are the product, as far as can now be determined, of causes acting upon the M A virus from within, that is, the result is due to internal rather than to external effects. In the

<sup>15</sup> Gotschlich, E., in Kolle-Wassermann, *Handbuch der pathogenen Mikroorganismen*, 2d edition, Jena, 1911, i, 166, 167.

course of the long propagation of the M A virus through monkeys the species was constant and the methods employed for inoculation remained uniform. These causes, whatever their nature, operate to produce a cycle of activity indicated by rise, fixation and fall in infecting power. And this is the cycle, apparently, that many epidemics pass through in the course of their appearance and disappearance.

There exists another fact inherent in all epidemics which lies, however, outside the present consideration, namely, the varying number of susceptible persons who fall victims to the prevailing disease. Our observations bear upon the conditions which make epidemics possible rather than those which determine their actual extent.

In the light of this presentation the part played by sporadic and abortive cases and of the microbe carriers of potentially epidemic diseases becomes more comprehensible. We may consider this class of infected persons or animals as carrying specific microorganisms lacking high virulence for their respective kind. And we may begin to see how the conversion, through favoring causes, of microorganisms of low into others of high virulence, may be the signal for the appearance of epidemics, not necessarily confined to one place but, possibly, arising almost simultaneously in separated and even remote places when the conditions are similar; just as, on the other hand, the immediate transportation of already elevated microorganisms from a place in which an epidemic is already prevailing to new places may start similar severe outbreaks there.

#### SUMMARY.

A strain of the poliomyelitic virus was propagated in monkeys for four years, during which time it displayed three distinct phases of virulence. The several phases covered different periods of time. At the outset the virulence was low, but by animal passages it quickly rose to a maximum; this maximum was maintained for about three years, when, without known changes in the external conditions, a diminution set in and increased until at the expiration of a few months the degree of virulence about equalled that present

at the beginning of the passages in monkeys. The cycle of changes in virulence is correlated with the wave-like fluctuation in epidemics of disease which also consist of a rise, temporary maximum, and fall in the number of cases prevailing. And an explanation of epidemics of disease is inferred in variations or mutations among the microorganismal causes of disease affecting chiefly the quality of their virulence.

## A CONTRIBUTION TO THE PATHOLOGY OF EPIDEMIC POLIOMYELITIS.\*

By SIMON FLEXNER, M.D., PAUL F. CLARK, PH.D., AND  
HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In the course of our studies on experimental poliomyelitis we have collected certain data on the pathology of the disease which seem of sufficient interest to warrant publication. They relate to the distribution of the virus in the nervous tissues outside the cerebrospinal axis and its bearing on the pathogenesis of poliomyelitis, the resistance of the virus to glycerin, phenol, and freezing, and to immune bodies in the cerebrospinal fluid after recovery from infection.

### THE VIRUS IN THE SPINAL, GASSERIAN, AND ABDOMINAL SYMPATHETIC GANGLIA.

That the virus of poliomyelitis is not present exclusively in the affected regions of the spinal cord but occurs also constantly in the brain was observed early in the course of the experimental inoculations of monkeys.<sup>1</sup> Later the virus was found to be present in the intervertebral ganglia of infected monkeys.<sup>2</sup> But no systematic study has been made from this point of view of the nervous organs that lie outside of and at a distance from the cerebrospinal axis. This we have now done with the results to be described, which have a bearing on the prevailing views on the pathogenesis of poliomyelitis.

The inoculation tests prove that the virus exists in the intervertebral, Gasserian, and abdominal sympathetic ganglia. Illustrative protocols follow of several positive inoculation experiments.

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<sup>1</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1639.

<sup>2</sup> Flexner, S., and Clark, P. F., *Jour. Am. Med. Assn.*, 1911, lvii, 1685.

*Protocol 1. Intervertebral Ganglia from the Monkey.*—Feb. 2, 1911. Several intervertebral ganglia were removed aseptically from a monkey that had been inoculated intracerebrally with M A virus and become paralyzed. The ganglia were emulsified in 2 c.c. of salt solution and injected intracerebrally into a *Macacus rhesus*. Feb. 7. Back muscles weak. Feb. 8. Extremities and back paralyzed. Feb. 9. Death. Microscopical examination of the spinal cord showed typical lesions of poliomyelitis.

*Protocol 2. Intervertebral Ganglia from the Monkey.*—Dec. 26, 1913. Several intervertebral ganglia were removed aseptically from a monkey that succumbed to inoculation of K virus. An emulsion in salt solution was injected into the sciatic nerve and peritoneal cavity. Jan. 1. A. M. Excitability; ataxia. P. M. Legs paralyzed. Jan. 2. Prostrate, died. Lesions of poliomyelitis.

*Protocol 3. Intervertebral Ganglia from a Child.*—Aug. 8, 1911. Several spinal ganglia which had been taken from a fatal case of acute poliomyelitis in a child and placed in glycerin a few days previously were emulsified in salt solution and injected intracerebrally into a *Macacus rhesus*. Aug. 19. Excitability; paralysis of right arm. Aug. 25. Paralysis of left leg. Sept. 1. Condition improving. Finally recovery occurred, with residual paralysis of one arm.

*Protocol 4. Gasserian Ganglia from the Monkey.*—Nov. 15, 1913. Four sets of Gasserian ganglia were removed from monkeys dead of K virus. They were emulsified in salt solution and injected into the right sciatic nerve and the peritoneal cavity of a *Macacus rhesus*. Dec. 2. Excitability; tremor; paralysis of right leg. Dec. 3. Both legs paralyzed; back weak; protects right arm. Dec. 4. Both arms weak. Dec. 5. Prostrate. Etherized. Lesions of poliomyelitis.

*Protocol 5. Sympathetic Ganglia from the Monkey.*—Nov. 15, 1913. Four sets of the abdominal sympathetic ganglia (solar plexus) removed from monkeys dead of K virus (same as preceding) were emulsified in salt solution and injected into the left sciatic nerve and the peritoneal cavity of a *Macacus rhesus*. Nov. 26. Excitability; weakness; paralysis of left leg. Nov. 27. Left arm weak; right arm weaker. Nov. 28. Left arm paralyzed; back weak. Nov. 29. Prostrate. Etherized. Lesions of poliomyelitis.

*Protocol 6. Sympathetic Ganglia from the Monkey.*—Dec. 3, 1913. Sympathetic ganglia were removed from a monkey succumbing to K virus and suspended in salt solution which was injected into the left sciatic nerve and peritoneal cavity. Dec. 9. Excitability. Left arm weak, and later in the day paralyzed. Dec. 11. Weakness of back. Dec. 12. Left leg paralyzed; right leg weak; right arm weak. Dec. 14. Prostrate. Etherized. Lesions of poliomyelitis.

The protocols show that the ganglia inoculated contain the active virus of poliomyelitis. In view of the frequency with which the demonstration can be made with the ganglia and the rarity with which it has been made with non-nervous tissues, with possibly the one exception of the mesenteric lymph nodes, we may conclude again that the virus is strongly neurotropic. Apparently the virus is not stored in the peripheral nerves<sup>3</sup> but seeks the parenchymatous nerv-

<sup>3</sup> Landsteiner, K., in Kolle-Wassermann, *Handbuch der pathogenen Mikroorganismen*, 2d edition, Jena, 1913, viii, 445.

ous organs in which to multiply. But the selection among the latter appears to be small, since the virus occurs regularly not only in the spinal cord but in the brain and the several ganglia mentioned, of which some are remote from the cerebrospinal axis.

Moreover, the localization of the virus in the ganglia takes place early. Monkeys in the preparalytic stage of infection, before any detectable weakness of muscles has set in but while in the state of excitement, already show lesions of the intervertebral ganglia. Thus far the other ganglia have not been studied in the early period of the infection.

The histological lesions in the ganglia correspond accurately with those in the spinal cord: they are partly interstitial and perivascular, partly parenchymatous. The former extend in part from the pial investment of the ganglia to the fibrillar supporting tissue and small blood vessels which may be the seat of diffuse or of nodular accumulations of mononuclear cells. The degree to which the nerve cells are destroyed is variable. At times they escape entirely even when the interstitial changes are marked; again they are widely degenerated, or even necrotic, and have become invaded with neurophages. So far as the changes in the spinal ganglia are concerned they appear to arise through an extension of the cellular invasion from the subarachnoid spaces of the spinal cord. The changes in the Gasserian are less severe than those of the spinal ganglia, and the mode of invasion is not so evident. The cellular accumulations within the abdominal ganglia (solar plexus) are least marked and so constantly perivascular that infection by way of the blood is indicated. The nerve cells exhibit the slightest lesions of all the ganglia studied.

In view of the constancy with which the sensory ganglia are the seat of early and even profound histological changes undoubtedly caused by the presence of the virus, it becomes questionable whether any considerable support still exists for the belief that poliomyelitis is essentially a disease of the anterior grey matter of the spinal cord.<sup>4</sup> It would appear rather to be a disease of the nervous system in its entirety, although considered clinically it receives its significance from the muscular paralysis that is caused. Nor can it be held that

<sup>4</sup> Landsteiner, K., *loc. cit.*, p. 439.

the virus of poliomyelitis possesses a special affinity for nerve cells as such, since the lesions which it causes are impressed almost indifferently upon nerve cells and non-nervous elements within the nervous organs. Indeed, accurate studies of the cerebrospinal fluid in preparalytic and abortive human cases of poliomyelitis emphasize the early involvement of the leptomeninges in the pathological process.<sup>5</sup>

The experiments in which intrasciatic injections were made indicate that the virus travels to the spinal cord by the lymphatic channels and brings about paralysis first on the side of injection and later on the opposite side.<sup>6</sup> It is interesting to find that the virus may ascend by means of the lymphatics of the cord to the cervical level and set up paralysis in an arm before the leg into which the virus was injected shows distinct evidences of paralysis (protocol 6).

#### RESISTANCE OF THE VIRUS TO GLYCERIN, PHENOL, AND FREEZING.

*Glycerin.*—It was early noted that the virus of poliomyelitis resists glycerin for a time.<sup>7</sup> Römer and Joseph<sup>8</sup> found specimens still active after five months' glycerination. We have tested a specimen of virus K and found that it retained its activity undiminished after twenty-five months in 50 per cent. glycerin, the preparation having been kept constantly in the refrigerator at about 4° C.

*Protocol.*—Sept. 24, 1913. A *Macacus rhesus* was inoculated intracerebrally with an emulsion of spinal cord containing virus K, which was derived from a paralyzed monkey on Aug. 21, 1911. On Oct. 1, or seven days after the inoculation, the animal was excitable, and the arms and back were weak. Oct. 2. Paralysis of extremities and back complete. Etherized. Lesions of experimental poliomyelitis.

The M A strain withstands glycerination probably as well as the K strain. A *Macacus* inoculated with an emulsion prepared from nervous tissue in glycerin for eight and eleven months respectively developed paralysis in characteristic manner.

<sup>5</sup> Draper, G., and Peabody, F. W., *Am. Jour. Dis. Child.*, 1912, iii, 153.

<sup>6</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1909, liii, 1913.

<sup>7</sup> Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

<sup>8</sup> Landsteiner, K., and Levaditi, C., *Compt. rend. Acad. d. sc.*, 1910, cl, 131; *Ann. de l'Inst. Pasteur*, 1910, xxiv, 833. Römer, P. H., and Joseph, K., *München. med. Wchnschr.*, 1910, lvii, 1059.

**Phenol.**—That the poliomyelic virus withstands phenolization was shown by Kraus,<sup>9</sup> who proposed first to treat paper filtrates of the infected spinal cord of monkeys with 0.5 per cent. and later with 1 to 1.5 per cent. solutions of phenol in order to diminish their potency and thus to make possible their employment for purposes of active immunization. Since the inoculations of the phenolized virus were made exclusively into subcutaneous tissues no index of the degree of its infectiousness was obtained. Macacus monkeys react irregularly to subcutaneous injections of the virus even when highly active. Flexner and Clark<sup>10</sup> employed phenol for the purpose of rendering bacteria-free the virus contained in tonsillar and adenoidal tissues, and to prepare them for inoculation into monkeys. They observed no injurious action of 0.5 per cent. phenol in the virus at the expiration of fourteen days' exposure. The long survival of the virus in undiminished activity in glycerin suggested the testing for activity of a specimen of human spinal cord and brain put aside in the refrigerator fifteen months before in 0.5 per cent. phenol.

**Protocol.**—Oct. 20, 1913. A *Macacus rhesus* was inoculated into the sciatic nerve and peritoneal cavity with an emulsion obtained from the central nervous tissues from an actively fatal case of poliomyelitis in a child, and preserved since July 25, 1912, in 0.5 per cent. phenol. Nov. 5. Paralysis of legs and weakness of back; arms strong. Nov. 6. Prostrate. Etherized. Lesions of poliomyelitis present.

Two experiments were made to determine whether 0.5 per cent. phenol acting upon the virus contained within a Berkefeld filtrate almost devoid of coagulable protein would affect the activity of the virus. The mixtures were allowed to remain at 22° C. for twenty hours and five days respectively, at the expiration of which period both were found to be highly infectious and capable of causing experimental poliomyelitis after average incubation time.

**Freezing.**—In contrast to the long survival of the virus in an active state in glycerin and phenol is its shorter survival when the tissues containing it are kept continually frozen at a temperature of —2° to —4° C. Under these conditions we have found the virus

<sup>9</sup> Kraus, R., *Wien. klin. Wchnschr.*, 1910, xxiii, 233; *Ztschr. f. Immunitätsforsch.*, 1911, ix, 117.

<sup>10</sup> Flexner, S., and Clark, P. F., *Jour. Am. Med. Assn.*, 1911, lvii, 1685.

active at the end of six weeks; but infection did not occur when material frozen for one and a half to three years was employed for inoculation.

*Neutralization Tests with Cerebrospinal Fluid.*—After recovering from experimental poliomyelitis monkeys are not subject to reinoculation; an active immunity has developed.<sup>11</sup> This state of immunity is associated with the occurrence in the blood of principles that neutralize the virus *in vitro*.<sup>12</sup> Since the poliomyelic virus produces profound alterations in the cerebrospinal fluid and this fluid gains ready access to the interstices of the central nervous tissues, tests were carried out to determine whether neutralizing antibodies existed within it. It is known that antibodies are not secreted in appreciable quantities into the cerebrospinal fluid; hence any considerable quantity that might be present there would probably have been produced locally.<sup>13</sup> The fluids selected for the tests were obtained from patients who had recently suffered from typical paralytic poliomyelitis from which they were convalescent, and were scrupulously free from blood. The acute symptoms had begun from one to three months prior to the collection of the cerebrospinal fluid.

Seven fluids were tested. The experiments were practically uniform. 0.1 of a cubic centimeter of highly active filtrate was mixed with from two to three cubic centimeters of the cerebrospinal fluid, the mixtures were incubated for two hours at 37° C., and placed in the refrigerator over night. The inoculations were intracerebral. Six of the monkeys developed typical paralysis after the average incubation period. One monkey remained perfectly well, from which it may be concluded that neutralization of the virus was accomplished. The child from whom the neutralizing specimen was obtained had been attacked about six weeks before. It would therefore appear possible, but highly unusual, for neutralizing immunity principles to be present in the cerebrospinal fluid during convalescence from epidemic poliomyelitis.

<sup>11</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1910, liv, 45.

<sup>12</sup> Levaditi, C., and Landsteiner, K., *Compt. rend. Soc. de biol.*, 1910, lxviii, 311.

<sup>13</sup> Flexner, S., *Jour. Am. Med. Assn.*, 1913, lxi, 447.

SUMMARY.

The virus of poliomyelitis is neurotropic, and localizes, and probably is capable of multiplying in the extramedullary parenchymatous nervous organs. It has been demonstrated by inoculation tests in the intervertebral, Gasserian, and abdominal sympathetic ganglia.

All the ganglia show histological lesions, more or less severe, similar to those of the spinal cord and brain. The severest occur in the intervertebral ganglia, those next in severity in the Gasserian, while the mildest appear in the abdominal sympathetic ganglia. The interstitial lesions predominate over the parenchymatous, and in preparalytic stages the intervertebral ganglia show interstitial lesions, especially pronounced at the pial covering.

Epidemic poliomyelitis is a general disease of the nervous system, although the most prominent and important symptoms are those following injury to the motor neurones of the spinal cord and brain.

The virus of poliomyelitis is highly resistant to glycerin, in which it survives for more than two years; to 0.5 per cent. phenol, in which it survives for more than one year; while it succumbs after having been kept frozen constantly for several months.

It is unsafe to employ phenol to modify the virus of poliomyelitis for the purpose of active immunization.

The cerebrospinal fluid of convalescents tends to be devoid of the neutralizing immunity principles for the virus of poliomyelitis, although they may exceptionally be present within this fluid. Doubtless the immunity principles are not produced locally in the nervous tissues, but elsewhere in the body, and are carried to the nervous organs by the blood.

## A NOTE ON THE ETIOLOGY OF EPIDEMIC POLIOMYELITIS.\*

BY HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

### PLATE 35.

Flexner and Noguchi<sup>1</sup> have cultivated a minute microorganism from the central nervous organs of human beings and monkeys that have succumbed to epidemic poliomyelitis, and Noguchi has perfected a method by which the parasite may be stained in film preparations and sections prepared from infected tissues. Either because of their minute size or because of the manner of distribution, the demonstration of the stained microorganisms in sections of the nervous tissues has been accomplished with difficulty. Moreover, one cannot be sure of the presence of a single organism in tissues where so many granules confuse the microscopic picture. I have therefore endeavored to arrive at more certain results by employing for the sections to be stained portions of nervous tissues which have previously been incubated for a sufficient time to allow the parasites to increase in number.

For the cultivation of the microorganism described by Flexner and Noguchi fragments of the brain are placed in sterile ascitic fluid together with a piece of sterile rabbit kidney, the whole being overlaid with paraffin oil. At the expiration of ten days the fluid culture is examined for growth. When uncontaminated with extraneous bacteria the medium shows almost no changes; and it may even remain unchanged when certain contaminating bacteria are present. The extraneous bacteria are detectable (a) by microscopical examination and (b) by subculture in the usual culture media. The smaller microorganism thus far cultivated only from poliomyelic tissues is readily distinguished from ordinary bacteria.

\* Received for publication, December 15, 1913.

<sup>1</sup> Flexner, S., and Noguchi, H., *Jour. Exper. Med.*, 1913, xviii, 461.

But many of the cultures set up as above stated remain permanently free from stainable microorganisms within the fluid. To obtain initial growth of the minute parasite requires much care and patience. The fragment of brain retains its form for many weeks and autolyzes slowly.

By this method of cultivation it is supposed that the minute microorganism first multiplies in the fragment of brain from which it gradually enters the ascitic fluid. It is probable therefore that growth may take place within the brain tissue and not in ascitic fluid, where for many reasons the conditions happen not to be favorable for multiplication. Since the parasite is subject to autolysis it may escape detection in microscopical preparations, because the small number that have entered from the brain tissue may have perished, or they may be too greatly diluted to be readily found.

However, it should be possible to detect the multiplied microorganism in the fragment of brain tissue itself. As a matter of fact this has been accomplished, and the minute parasites have been found in such states as to show conclusively that they have multiplied post mortem.

Flexner and Noguchi pointed out that the minute globoid bodies which they cultivated appeared within the nervous tissues in single and double form, but not in chains or distinct masses such as occur in artificial cultures. We may assume therefore that during post-mortem multiplication within the tissues both the chain and mass form would arise, as has been actually observed. The procedure employed for the demonstration of the bodies in the incubated tissue follows.

*Monkey A.*—Inoculated intracerebrally on the left side with 0.3 c.c. of a Bérkefeld filtrate of M A virus, began to show symptoms of poliomyelitis on the seventh day, and ran a typical course. The animal became prostrate on the ninth day and was etherized on the thirteenth. Small portions (about 1 by 1 by 2 cm.) of the right brain were placed in tubes of kidney-ascitic fluid culture medium and incubated at 37° C. Beginning with the second day and daily thereafter a tube was taken out and the fluid replaced by molten agar, and the mass containing the piece of brain was poured immediately into a small dish. When the agar had congealed small pieces of the brain were cut and dropped into pyridin-lucidol<sup>2</sup> for hardening, according to the method described by Szésci, and finally blocked

<sup>2</sup>Lucidol is the name given by Szésci to benzoyl peroxide. Szésci, St., *Deutsch. med. Wchnschr.*, 1913, xxxix, 1584.

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organisms multiply within the nervous tissue when incubated in this fluid culture medium. The usual procedure for obtaining cultures would be to allow them to multiply and then set them free by some means into the fluid.

Monkeys brains known to contain K virus were incubated in the kidney-ascitic fluid culture medium. Examinations by smear preparations of the fluid at intervals of time up to fifty days gave no evidence that a culture had been obtained.

The pieces of nerve tissue were then crushed by means of a glass rod and placed in tubes incubated for eleven days more, when the organisms were found in pure culture. The organisms grew in subcultures from these tubes.

Incubation for twelve days before crushing the bits of brain, and then incubation for three days before making subcultures is sufficient to obtain good growths.

Flexner and Lewis<sup>4</sup> have shown that the virus of poliomyelitis can be demonstrated in the circulating blood, and recent experiments by Flexner, Fraser, and Amoss confirm and extend the earlier work obtained by Flexner and Lewis. On the other hand, the investigations are that the virus is not present in the blood either abundantly or constantly. An effort was therefore made to detect the organism in blood films with a successful result in one instance.

*Monkey C. Macacus rhesus.*—Inoculated intraspinously with 1 c.c. of a suspension of K virus, ran a typical course, and became prostrate in ten days. The animal was etherized on the twelfth day and film preparations were made from the heart's blood. These were fixed in methyl alcohol and stained for two hours in 1:20 Giemsa solution and decolorized by standing in acetone for eight hours. Two short extracellular chains were found, each consisting of four globules identical in morphology and staining properties with the previously described organism. One of these sets is shown in figure 5.

*Monkey D. Macacus rhesus.*—Received intravenously 180 c.c. of a Berkeley filtrate of K virus. Eleven days later typical poliomyelitis ensued and the monkey was moribund on the fourteenth day, when it was etherized and 2 c.c. of the heart's blood were placed into kidney-ascitic culture medium. Fifteen days later film preparations did not disclose the presence of the organism, but transplants were made and the organism was found in pure culture after twenty days' incubation.

#### SUMMARY.

The globoid bodies, or minute microorganisms, cultivated from the central nervous organs of human beings and monkeys that have succumbed to poliomyelitis, may be detected in the incubated brain tissues of infected monkeys in forms indicating post-mortem multiplication. Incubating the poliomyelitic tissues in kidney-ascitic fluid culture medium and then crushing them is a more certain method for obtaining cultures of the organism.

Identical bodies have been detected in blood films prepared on the twelfth day of the acute attack, from a paralyzed poliomyelic monkey inoculated intraspinously.

The same organism has been cultivated from the blood of a monkey that had received intravenously a large dose of a Berkefeld filtrate of poliomyelic virus.

No other microorganisms were detected either in the sections of the brain or in film preparations of the blood. These observations tend therefore to confirm the etiological relationship between the minute microorganism and epidemic poliomyelitis suggested by the successful cultivation and inoculation experiments reported by Flexner and Noguchi.

#### EXPLANATION OF PLATE 35.

FIG. 1. Globoid bodies in chain formation in brain tissue after six days' incubation.  $\times 1,000$ .

FIG. 2. Globoid bodies in chain formation in brain tissue after eight days' incubation.  $\times 1,000$ .

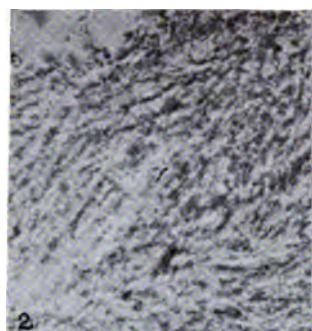
FIG. 3. Globoid bodies in chain formation in brain tissue after ten days' incubation.  $\times 1,000$ .

FIG. 4. Globoid bodies in mass formation in brain tissue after thirty days' incubation.  $\times 1,000$ .

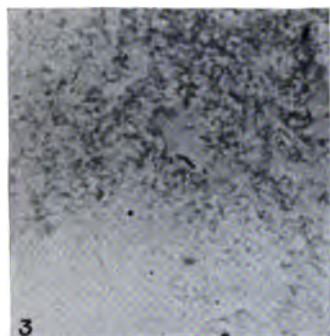
FIG. 5. Globoid bodies in chain formation in heart's blood.  $\times 1,000$ .



1



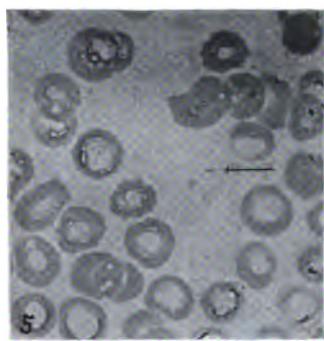
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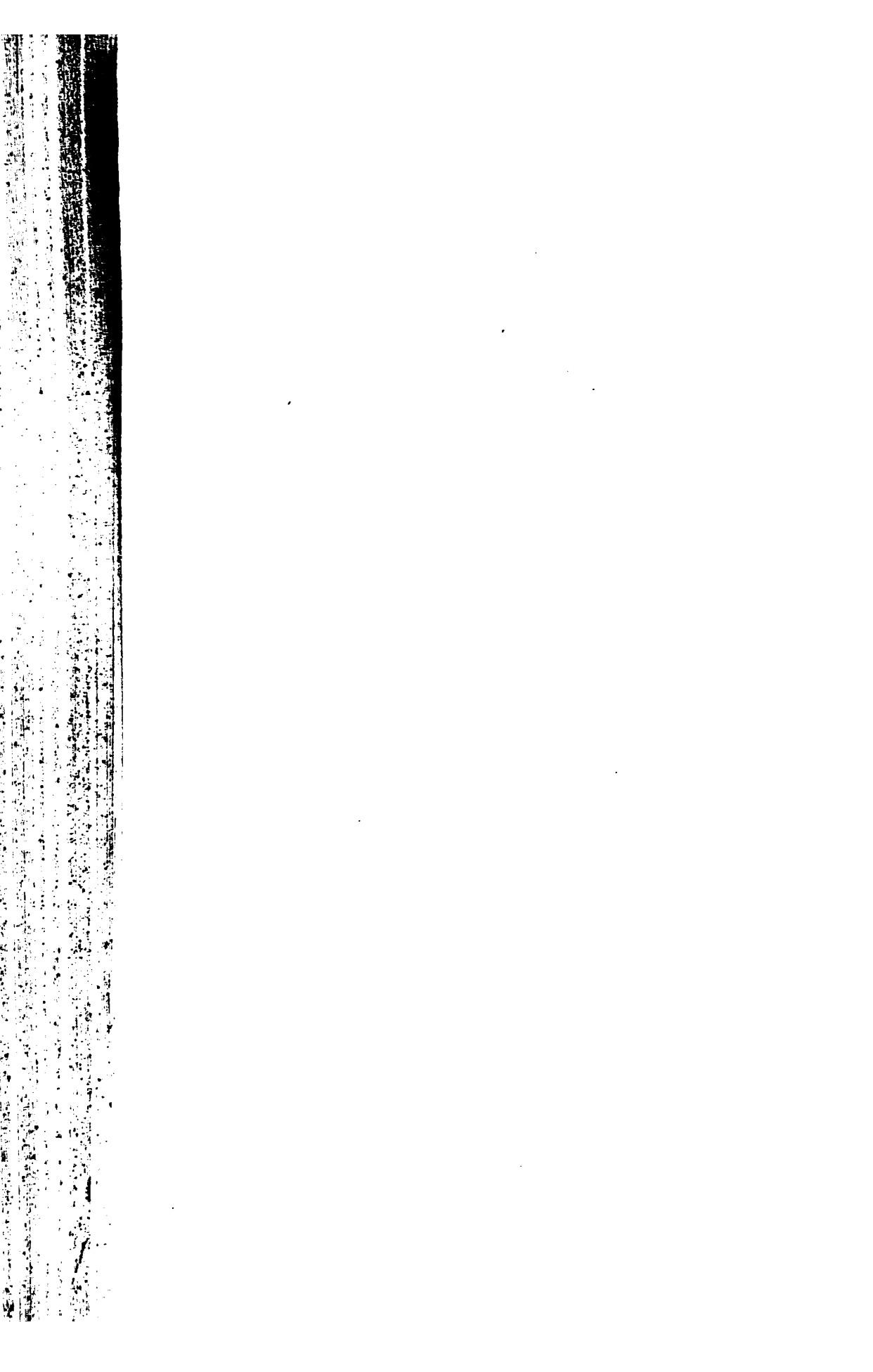


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(Amoss: Etiology of Epidemic Poliomyelitis.)



ROBERT BROWN  
HOSPITAL

## INTRASPINOUS INFECTION IN EXPERIMENTAL POLIOMYELITIS.\*

By PAUL F. CLARK, PH.D., AND HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The routes by which the virus of poliomyelitis may be conveyed to the central nervous organs of monkeys so as to induce infection and paralysis are various but not of equal certainty. Undoubtedly the direct intracerebral injection yields the most constant results, next to which the intrasciatic and intranasal have been placed. Infection by way of the peritoneal cavity, subcutaneous tissues, and blood is obtained with far less certainty. Considerable interest attaches to the intraspinous route of inoculation of the virus, because of the bearing which it may have on the theory of the pathogenesis of poliomyelitis.

That the virus of poliomyelitis exhibits a great affinity for the nervous organs is obvious from the location of the main lesions of the disease. There is, however, lack of agreement as to the manner in which the lesions are produced; namely, whether through direct action of the virus upon the nerve cells or through indirect effect of lesions in the blood vessels and ground substance. The hypothesis that poliomyelitis is a specific affection of the anterior grey matter of the spinal cord has been abandoned. The lesions occur regularly throughout the structures of the cord including the intervertebral ganglia, frequently in the medulla and brain, and quite often in the Gasserian and abdominal sympathetic ganglia.<sup>1</sup> Probably other ganglionic masses, not yet studied, will show effects. Besides, lesions are present in the lymphatic and other somatic organs.<sup>2</sup>

\* Received for publication, December 15, 1913.

<sup>1</sup> Flexner, S., Clark, P. F., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 205.

<sup>2</sup> Flexner, S., Peabody, F. W., and Draper, G., *Jour. Am. Med. Assn.*, 1912, lviii, 109.

The pia-arachnoid membranes of the central nervous system are constantly involved in the pathological process, although the degree of involvement varies. Examination of the cerebrospinal fluid in human cases regularly shows an increase in cells and globulin, thus indicating an inflammation of the meninges.<sup>3</sup> Histological examination of the spinal cord of fatal human and experimental cases shows cellular infiltration of the pial membrane, particularly adjacent to the surface of the spinal cord and most marked near the blood vessels of the membrane. The pia projecting into the anterior and in a less degree into the posterior median columns contains this cellular infiltration, which, within the grey substance of the spinal cord, may be strongly marked about the blood vessels and even in the ground substance. While some degree of infiltration is never absent, cases in man and in the monkey occur in which it is slight. A similar infiltration exists in the intervertebral ganglia appearing to extend from the pial investment into their substance. Along with the cellular infiltration are nerve cell degenerations of various grades. Sometimes the infiltrations predominate in degree over these degenerations, sometimes the degenerations predominate over the infiltrations.

Flexner has emphasized the nasopharyngeal mode of infection in poliomyelitis, and according to his view the virus ascends probably by way of the lymphatics from the nasal mucous membrane and multiplies in the pia-arachnoid membranes and the adjacent nervous structures of the brain before becoming established in the medulla and spinal cord.<sup>4</sup> Hence it has seemed important to ascertain whether infection can be readily produced by introducing the virus directly into the subarachnoid spaces. This may be done by a subdural injection into the region of the brain after trephining or into that of the spinal cord by means of lumbar puncture. The latter is the simpler and safer method, as there is little risk of introducing the fluid into the nervous tissues themselves.

Thus far Flexner and Lewis<sup>5</sup> alone seem to have recorded a suc-

<sup>3</sup> Peabody, F. W., and Draper, G., *Am. Jour. Dis. Child.*, 1912, iii, 153.

<sup>4</sup> Flexner, S., *Jour. Am. Med. Assn.*, 1910, iv, 1105; Huxley Lecture, *Lancet*, 1912, ii, 1271, and *Science*, 1912, xxxvi, 685.

<sup>5</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1910, liv, 535.

cessful infection among several failures by means of intraspinous inoculation of the virus, although Neustaedter and Thro caused poliomyelitis in three monkeys by means of combined intraspinous and subcutaneous inoculation,<sup>6</sup> and Römer,<sup>7</sup> without giving any experimental details, remarks that intraspinous infection takes place readily.

No special difficulty surrounds the intraspinous mode of infection. The requirements are an active virus, such as our M A and K strains, and a dose somewhat larger than that necessary for successful intracerebral inoculation. However, the immediate effects of the two modes of injection are different. When the virus is injected into the brain it is usually deposited in a small cavity within the cerebral hemisphere although some may escape into the meninges. It may safely be assumed that multiplication of the virus soon begins under the favorable conditions of temperature and location, and that gradually it passes into the adjacent membranes and general nervous tissue in which further multiplication occurs. When the virus is injected into the subarachnoid spaces in the lumbar region it quickly diffuses in the membranes along the length of the spinal cord and base of the brain, but it begins also immediately to pass with the cerebrospinal fluid into the circulating blood through the usual venous channels. Since the blood route is perhaps the poorest for producing infection the virus which escapes from the subarachnoid spaces into the veins may be regarded as lost.

Hence, instead of Berkefeld filtrates we have found it better to employ paper filtrates of the usual 5 per cent. emulsion of the infected nervous tissue containing the virus. In a series of nine monkeys inoculated by lumbar puncture with paper filtrates of M A virus, all developed poliomyelitis. Of this series six monkeys became paralyzed, while three developed definite symptoms without showing paralysis. The microscopical examination of the spinal cord and intervertebral ganglia of the latter three brought out the presence of typical poliomyelic lesions of mild degree.

<sup>6</sup> Neustaedter, M., and Thro, W. C., *New York Med. Jour.*, 1911, xciv, 813.

<sup>7</sup> Römer, P. H., *Die epidemische Kinderlähmung*, Berlin, 1911, 132.

## ILLUSTRATIVE PROTOCOLS.

*Experiment 1. Macacus rhesus.*—Feb. 18, 1913. A small quantity of cerebrospinal fluid was withdrawn by lumbar puncture and 1 c.c. of a paper filtrate from a 5 per cent. suspension of the spinal cord from a recently paralyzed M. A monkey was injected. Feb. 22. Tremor; left arm paralyzed; extremities weak. Feb. 23. Paralysis of arms, back, neck, and right leg. Feb. 24. Prostrate. Etherized. Characteristic lesions of experimental poliomyelitis present in the spinal cord and intervertebral ganglia.

*Experiment 2. Macacus rhesus.*—Feb. 26. 0.5 c.c. of cerebrospinal fluid was withdrawn by lumbar puncture, and 0.5 c.c. of a paper filtrate prepared from a recently paralyzed animal was injected. Mar. 3. Weakness of left arm. Mar. 7. Extremities paralyzed; prostrate. Mar. 8. Etherized. Lesions of poliomyelitis present.

*Experiment 3. Macacus rhesus.*—May 22. Cerebrospinal fluid was withdrawn by lumbar puncture, and 1.2 c.c. of a paper filtrate prepared from a recently paralyzed animal was injected. May 29. Excitable; tremor. May 30. Legs weak. May 31. Legs and arms weak. June 2. Excitability increased; neck weak. June 3. Weakness of arms and legs increased. June 7. Condition stationary. Etherized. The spinal cord and intervertebral ganglia showed typical but mild lesions of poliomyelitis.

The protocols given serve as examples of the remaining experiments. From them it can be concluded that given a specimen of virus of adequate virulence experimental poliomyelitis can be regularly produced by intraspinous inoculation.

The virus having been introduced into the subarachnoid space clearly gains access to the nervous tissues of the spinal cord and medulla, with the interstices of which the cerebrospinal fluid is in intimate communication. A part, therefore, becomes fixed to the tissues within the pial membrane and about the blood vessels and doubtless to the nervous structures proper. Another part readily leaves the subarachnoid spaces with the cerebrospinal fluid. Thus far no one has detected the virus in the cerebrospinal fluid in human cases of poliomyelitis and it is usually absent from the fluid in monkeys at the time of the onset of paralysis, although it may be present at an earlier period after intracerebral inoculation.<sup>8</sup> We have, therefore, tested the cerebrospinal fluid at intervals of twenty-four and forty-eight hours after the inoculation, and at the expiration of six days when the first symptoms of paralysis made their appearance.

<sup>8</sup> Flexner, S., and Lewis, P. A., *Jour. Am. Med. Assn.*, 1911, lvii, 1685.

*Macacus rhesus A.*—Nov. 22. Intraspinous injection of 3 c.c. of a paper filtrate of K virus was given after withdrawal of 2 c.c. of clear cerebrospinal fluid. Nov. 23. 0.8 c.c. of turbid fluid was withdrawn by lumbar puncture. Nov. 24. 1 c.c. of less turbid fluid. The fluids were inoculated intracerebrally into rhesus monkeys. Nov. 26. Excitable; legs paralyzed; back weak. Nov. 27. Prostrate. Died. Typical poliomyelitic lesions present in the spinal cord.

*Macacus rhesus B.*—Nov. 23. Intracerebral injection made of the 24 hour specimen of fluid withdrawn by lumbar puncture from monkey A.

Nov. 30. Excitable. Dec. 2. Paralysis of left leg. Dec. 4. Muscles of extremities and back paralyzed; prostrate. Dec. 5. Etherized. Lesions of poliomyelitis present.

*Macacus rhesus C.*—Nov. 24. Intracerebral injection of 1 c.c. of spinal fluid withdrawn by lumbar puncture from monkey A 48 hours after intraspinous inoculation.

Nov. 29. Excitable; right arm and leg paralyzed; back weak. Nov. 30. Prostrate. Dec. 2. Etherized. Lesions of poliomyelitis present.

*Macacus rhesus D.*—Nov. 22. 1 c.c. of a paper filtrate of the virus was given by intraspinous injection. Nov. 28. First symptom of infection. 2 c.c. of cerebrospinal fluid were withdrawn, and on Nov. 29 injected intracerebrally into another rhesus monkey, E. No symptoms developed in the latter.

These experiments show again, first, that infection by way of the subarachnoid spaces readily occurs, and next, that the virus even when introduced directly into them tends to escape from these spaces partly into the central nervous tissues, where it is held and in which it multiplies, and into the general circulation by which it is probably carried to all the nervous organs which doubtless again in some degree remove it from the blood. The reasons for failure to detect the virus in the cerebrospinal fluid in human cases are now apparent.

#### SUMMARY.

By intraspinous injections of specimens of poliomyelitic virus of suitable virulence infection can be caused regularly in *Macacus rhesus* monkeys.

The virus passes from the subarachnoid spaces into the nervous tissues in which it multiplies, and into the blood.

The constant involvement of the pia-arachnoid membranes in poliomyelitis, even when no paralysis occurs, and the fact that infection can readily be produced by intraspinous inoculation suggests anew that in the pathogenesis of poliomyelitis the interstitial tissue changes within the meninges, blood vessels, and ground substance play a determining part.

While the virus injected into the subarachnoid spaces can be demonstrated there by inoculation tests forty-eight hours after the injection it can no longer be detected on the sixth day, at a time when the first symptoms of infection make their appearance. The failure of the cerebrospinal fluid from human and experimental cases of poliomyelitis to produce the disease when inoculated into monkeys is due to the fact that the virus is either fixed by the nervous tissues or passes into the blood.

Mr. & Mrs.  
Horn

АМЕРИКАНСКАЯ  
ДИПЛОМАТИЯ

## THE RELATION TO THE BLOOD OF THE VIRUS OF EPIDEMIC POLIOMYELITIS.\*

By PAUL F. CLARK, PH.D., FRANCIS R. FRASER, M.B., AND  
HAROLD L. AMOSS, M.D.

(*From the Laboratories and Hospital of The Rockefeller Institute for Medical Research.*)

Early in the history of experimental poliomyelitis Flexner and Lewis<sup>1</sup> succeeded in one instance in producing infection in a Macacus monkey through the intravenous injection of twenty-five cubic centimeters of the defibrinated blood obtained from a recently paralyzed monkey. When a smaller quantity (ten cubic centimeters) of the blood was injected into the circulation, or still smaller quantities were introduced intracerebrally, paralysis did not follow. Leiner and von Wiesner<sup>2</sup> at first reported only negative results from the injection of blood from both human and experimental cases of poliomyelitis; but later<sup>3</sup> they observed in one instance paralysis following the injection, partly intracerebral and partly intravenous, of defibrinated blood taken from a monkey on the third day of paralysis. Römer<sup>4</sup> and Landsteiner and Levaditi<sup>5</sup> also record only failure both with the blood of human and of experimental cases of the disease.

The subject of the relation to the blood of the virus of poliomyelitis is of more than theoretical interest, as it may have a bearing on the manner of transmission of the disease. On that account we have carried out a larger series of experiments in order to determine, as far as possible with the methods at present available, what this relation is.

The first series of inoculations was performed with blood taken

\* Received for publication, December 20, 1913.

<sup>1</sup> Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

<sup>2</sup> Leiner, K., and von Wiesner, R., *Wien. klin. Wchnschr.*, 1909, xxii, 1698.

<sup>3</sup> Zappert, J., von Wiesner, R., and Leiner, K., *Studien über die Heine-Medinsche Krankheit*, Leipzig and Vienna, 1911, 171.

<sup>4</sup> Römer, P. H., *München. med. Wchnschr.*, 1910, lvii, 229.

<sup>5</sup> Landsteiner, K., and Levaditi, C., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 833.

## 224 Relation to the Blood of Virus of Epidemic Poliomyelitis.

from human cases admitted to the Hospital of The Rockefeller Institute.

### SURVIVING HUMAN CASES.

*Case 1.*—N. B. Age, 21 years. Onset Oct. 1, 1910. Condition: weakness and atrophy of muscles of right shoulder girdle and arm, atrophy of muscles of right thigh. Blood and spinal fluid normal. Blood collected 24/24<sup>6</sup>.

Oct. 25. 2 c.c. of defibrinated blood injected intracerebrally and 6 c.c. intraperitoneally into a *Macacus rhesus*. No symptoms of poliomyelitis developed.

*Case 2.*—J. H. Age, 12½ years. Onset Oct. 16, 1910. Condition: partial paralysis of legs; temperature 101.6° F. Oct. 27. Increased globulin in spinal fluid. Blood collected 13/9.

Oct. 27. Immediately after collection 3 c.c. of the whole blood were injected intracerebrally and 6 c.c. intraperitoneally into a *Macacus rhesus*. No effects followed.

*Case 3.*—J. A. Age, 20 years. Onset July 14, 1911. Condition: complete paralysis of right and partial paralysis of left leg. Increased cells and globulin in spinal fluid. July 22. Blood collected and defibrinated 9/7.

July 22. 4 c.c. of the blood were injected intracerebrally and 10 c.c. intraperitoneally into a *Macacus rhesus*. Monkey remained well.

*Case 4.*—M. B. Age, 5 years. Onset Oct. 3, 1912. Condition: temperature 104.8° F.; right leg paralyzed; left leg weak; neck and back stiff; facial muscles on left side weak. Increased cells in spinal fluid. Oct. 8. Right arm paralyzed. Blood collected 6/3.

Oct. 8. The whole blood was immediately injected as follows: 2 c.c. intracerebrally, and 30 c.c. intraperitoneally into a *Macacus rhesus*. No ill effects followed.

*Case 5.*—A. J. Age, 10 years. Onset Oct. 13, 1912. Condition: tenderness in limbs and back; left arm weak; right shoulder and extensors of right arm paralyzed; intercostal muscles on both sides paralyzed. Oct. 23. Blood collected 11/7.

Oct. 23. Injected 30 c.c. of whole blood into the femoral vein of a *Macacus rhesus*. Monkey remained well.

*Case 6.*—H. O'C. Age, 3 years. Onset Nov. 1, 1912. Condition: temperature 101.8° F.; right leg paralyzed; intercostal muscles on left side weak; muscles of anterior abdominal wall and anal sphincters weak. Increased cells in spinal fluid. Nov. 5. Blood collected 5/4.

Nov. 5. 2 c.c. of whole blood were injected into the left sciatic nerve of a *Macacus rhesus*. No symptoms of poliomyelitis developed.

### FATAL HUMAN CASES.

*Case 7.*—G. G. Age, 9½ years. Onset Aug. 23, 1911. Aug. 30. Condition: temperature 102.2° F.; legs completely, arms partially paralyzed; intercostal muscles completely, diaphragm partially paralyzed. Sept. 1. Death. Blood collected aseptically from the heart post mortem.

<sup>6</sup> In each case the numerator of the fraction denotes the day of the disease and the denominator the day of the paralysis on which the blood was collected from the arm vein for injection.

**Sept. 2.** Injected 3 c.c. of the defibrinated blood intracerebrally and 12 c.c. intraperitoneally. Monkey remained well.

**Case 8.**—J. L. Age, 5½ years. Onset July 12, 1912. July 20. Condition: temperature 105° F.; legs and muscles of shoulders paralyzed; facial muscles of right side weak; intercostal muscles paralyzed. Death same day. Blood collected aseptically from heart post mortem.

July 21. 30 c.c. of citrated blood were injected intraperitoneally into a *Macacus rhesus* monkey, and 0.75 c.c. intracerebrally and 30 c.c. intraperitoneally into another *Macacus rhesus*. Neither monkey developed symptoms of poliomyelitis.

**Case 9.**—S. N. Age, 6 years. Onset Sept. 28, 1912. Sept. 30. Condition: patient cyanotic and gasping for breath; intercostal muscles paralyzed and diaphragm weak. Increased globulin and cells in spinal fluid. Died same day. Blood collected aseptically from heart post mortem.

**Case 10.**—G. G. Age, 20 months. Onset gradual and indefinite. Sept. 30, 1912. Intercostals paralyzed; shoulder muscles and facial muscles on right side weak. Oct. 2. Death. Blood collected from heart post mortem.

**Case 11.**—M. K. Age, 2 years. Onset Sept. 28, 1912. Oct. 1. Temperature 102.4° F.; legs completely paralyzed; intercostals weak; pulse weak and irregular. Increased cells and globulin in spinal fluid. Oct. 4. Consolidation of both lungs. Temperature 104.5° F. Death on same day. Blood collected from heart post mortem.

Oct. 11. Injected 2.5 c.c. of the mixed defibrinated blood of these three fatal cases intracerebrally, and 7 c.c. intraperitoneally into a *Macacus rhesus* which showed excitability on Oct. 15. No paralysis occurred; and later a test inoculation with M A virus caused typical paralysis. Hence it was concluded that this monkey had not developed an abortive attack of experimental poliomyelitis from the injection of the blood.

**Case 12.**—J. C. Age, 9 years. Onset Oct. 5, 1912. Oct. 8. Condition: temperature 102.6° F.; muscles of right leg and both shoulder girdles paralyzed; intercostal muscles weak. Increased cells and globulin in spinal fluid. Oct. 9. Death. Blood collected from heart post mortem.

Oct. 9. 2 c.c. of the whole blood injected intracerebrally and 35 c.c. intraperitoneally into a *Macacus rhesus*. No effects followed.

These tests confirm those previously made by others with the blood from human cases of poliomyelitis. They do not, however, suffice to exclude altogether the possibility of the virus being present in the blood, since it is known that the original human strains are not as infectious for monkeys as the adapted strains. Another complicating factor may sometimes play a part in preventing infection from the blood. After the first week of acute illness immune bodies which have a neutralizing effect on the virus appear in the blood, and it is not improbable that by acting on the virus, originally of low infective power for monkeys, they may further diminish the chances of producing paralysis. In view of these considerations

we have turned to the experimental cases of poliomyelitis in order to find a solution of the question.

#### EXPERIMENTAL.¶

In several instances in the course of our experimental study of poliomyelitis we have made inoculations of the blood of monkeys in the acute stage of paralysis into the brain or peritoneum of normal monkeys in order to ascertain whether the virus is thus demonstrable in the circulating blood. They all resulted negatively.

The experiments were now modified as follows: heavy emulsions of the spinal cord taken from a recently paralyzed animal were injected (a) intracerebrally and (b) both intracerebrally and intraspinally into monkeys from which blood was taken at intervals for inoculation intracerebrally into other monkeys. It was reasoned that the virus would be carried from the subarachnoid spaces into the veins of the membranes of the spinal cord and thence into the general blood. The protocols follow.

*Experiment A.*—Mar. 24, 1913. 3 c.c. of a suspension of glycerinated M A spinal cord were injected intracerebrally into a *Macacus rhesus*. Blood was taken one, six, twenty-four, and forty-eight hours after inoculation. Mar. 31. Paralysis of arms and back. Apr. 2. Etherized. With the blood taken at the intervals given other monkeys received intracerebral injections of 1 c.c. each. None developed symptoms of poliomyelitis.

*Experiment B.*—May 16, 1913. A *Macacus rhesus* was injected intracerebrally with 2 c.c., and intraspinally with 5 c.c. of emulsion of the spinal cord (M A) taken from a paralyzed monkey. Blood was taken one, six, and twenty-four hours after inoculation for reinoculation into other monkeys. May 24. Paralysis and prostration; etherized. With the blood withdrawn at intervals, from 2 to 3 c.c. were injected intracerebrally into *Macacus rhesus* monkeys, none of which showed symptoms of poliomyelitis.

Hence under conditions favorable for the passage of the virus into the blood none could be detected by the experiments performed. However, as will appear later, the negative results obtained might have been due in part to the relatively weak virulence of the strain of virus employed since the M A virus had at this period lost a large part of its activity.<sup>7</sup> When a highly active virus is employed for intracerebral inoculation it has happened exceptionally that the

<sup>7</sup> Flexner, S., Clark, P. F., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 195.

blood is infective when taken early in the paralysis or at the onset of symptoms,<sup>8</sup> as is shown in the following protocol.

*Experiment C.*—A *Macacus rhesus* had been inoculated intracerebrally with K virus on Nov. 17, 1913, and on Nov. 24 had begun to show definite early symptoms of infection. Blood was immediately withdrawn and defibrinated, and 4 c.c. were injected intracerebrally into another rhesus monkey which on Nov. 29 was somewhat excitable. On Nov. 30 the latter showed paralysis of the left leg. On Dec. 1 the arms and back were weak. On Dec. 2 animal died. Typical lesions of poliomyelitis were present.

We next resorted to the active K virus for the double inoculation.

*Experiment D.*—Jan. 5, 1914. A needle was introduced into the lumbar cistern of the spinal cord of a *Macacus rhesus*, and a few drops of clear cerebrospinal fluid were permitted to escape. While in position 2 c.c. of an emulsion of K virus were injected intracerebrally. The injection was followed at first by the escape of clear fluid, and in two and a half minutes turbid blood-tinted fluid escaped from the lumbar puncture needle. On completion of the intracerebral inoculation 2 c.c. of the emulsion were injected intraspinally. Five hours and seventy-two hours later blood was taken from the median basilic vein and defibrinated for intracerebral inoculation. Jan. 8. Excitable; left arm and back weak; nystagmus. Jan. 10. Paralysis of arms. Jan. 12. Death. Lesions of poliomyelitis present.

Two *Macacus* monkeys were injected intracerebrally, one with the defibrinated blood taken five hours, and the other three days (at onset of paralysis) after the double inoculation. Both remained well.

This experiment indicates that the detection by inoculation of even a highly pathogenic virus in the blood following combined intracerebral and intraspinal injection is difficult and uncertain. That the virus may sometimes appear in the blood after intraspinal inoculation is shown by the detection of the globoid microorganisms in the film preparations in one such instance.<sup>9</sup>

The problem was now approached from another side. The virus was injected into the veins and the blood tested subsequently in order to ascertain whether it remains and multiplies there or is filtered out and removed. While doubt may exist as to whether the virus enters the blood in quantity and remains there in the case of intracerebral inoculation there can be no doubt of the entrance by the intravenous mode of inoculation, in which the quantity introduced may also be varied at will. Hence a series of experiments

<sup>8</sup> Zappert, J., Leiner, K., and von Wiesner, R., *loc. cit.*

<sup>9</sup> Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 212.

was made by injecting the virus intravenously and withdrawing blood at intervals from a distant vein and injecting it intracerebrally into normal animals.

*Experiment E. Macacus rhesus 1.*—Oct. 30, 1913, 3 P. M. 180 c.c. of a filtrate of K virus were injected into the right external saphenous vein. 4 P. M. Sufficient blood was withdrawn from the left external saphenous vein to yield 4 c.c. after defibrination. Oct. 31, 3 P. M. The same quantity was withdrawn from the left leg, and the defibrinated blood specimens were inoculated into two *Macacus rhesus* monkeys, 2 and 3. Nov. 9. Very weak; excitable. Died during night. Lesions of poliomyelitis present in spinal cord. Control died in six days. Typical lesions.

*Monkey 2.*—Oct. 30. One hour blood specimen inoculated intracerebrally. Nov. 9. Excitable and weak. Nov. 10. Left facial paralysis; ataxic. Nov. 12. Prostrate. Etherized. Lesions of poliomyelitis were present.

*Monkey 3.*—Oct. 31. 24 hour specimen inoculated intracerebrally. Nov. 10. Excitable; back and arms paralyzed. Nov. 11. Prostrate. Etherized. Lesions of poliomyelitis were present.

*Experiment F. Macacus rhesus 1.*—Nov. 14, 1913, 10.50 A. M. 100 c.c. of a filtrate of K virus were injected into a vein of the right leg. 12 M. Sufficient blood was withdrawn from a vein in the left leg to yield 4 c.c. after defibrination. Nov. 15, 11 A. M. Same quantity of blood withdrawn. Nov. 16, 11 A. M. Same quantity withdrawn. Nov. 17, 11 A. M. Same quantity withdrawn. Nov. 24 Excitable; left leg paralyzed; left facial paralysis. Sufficient blood withdrawn to yield 4 c.c. when defibrinated. Nov. 25. Legs, arms, and back weak or paralyzed. Dec. 1. Died. Lesions of poliomyelitis were present.

*Monkey 2.*—Nov. 14. One hour blood specimen was injected intracerebrally into a *Macacus rhesus*. Nov. 18. Excitable; left facial paralysis. Nov. 19. Extremities paralyzed. Nov. 20. Prostrate. Etherized. Lesions of poliomyelitis.

*Monkey 3.*—Nov. 15. 24 hour specimen inoculated intracerebrally. No symptoms developed.

*Monkey 4.*—Nov. 16. 48 hour specimen inoculated intracerebrally. Nov. 22. Excitable. Arms paralyzed. Nov. 24. Prostrate. Died. Lesions of poliomyelitis.

*Monkey 5.*—Nov. 17. 72 hour specimen inoculated intracerebrally. Nov. 24. Excitable. Arms and legs paralyzed. Blood was withdrawn for reinoculation, and employed in experiment C (page 227). Etherized. Lesions of poliomyelitis were present.

*Monkey 6.*—Nov. 24. 4 c.c. of defibrinated blood, taken 10 days after the intravenous injection of the filtrate when paralysis appeared, was inoculated intracerebrally into monkey 6. No symptoms appeared.

These experiments show clearly that when large and overwhelming quantities of an active filtrate are injected into the circulation the virus persists in the blood for seventy-two hours at least. Finally, it appears to be removed, as ten days after its injection, at the period of the onset of paralysis, it may not be detectable by

the inoculation test. The failure of the monkey inoculated with the twenty-four hour specimen in experiment F to respond may have been due to resistance in the animal rather than to absence of the virus; however, this animal when subsequently tested with an emulsion became typically paralyzed. That the ten day specimen of blood was no longer infectious is not remarkable in view of the many negative results from inoculations of blood at the beginning of the paralysis; but the successful result (experiment C, page 227) in which the blood was infectious at the onset of the paralysis seven days after an intracerebral injection shows that the active virus may pass into the blood from the nervous tissues in which it has multiplied and may survive there for a time.

The infectivity of the blood is affected by the amount of virus injected. Experiments were made also with intravenous injections of ten cubic centimeters of filtrate.

*Experiment G.*—Dec. 15, 1913. 10 c.c. of filtrate of active K virus were injected into the right external saphenous vein of a *Macacus rhesus*. Dec. 16 (24 hours later) and Dec. 17 (48 hours later) 5 c.c. of blood withdrawn from the left external saphenous vein were defibrinated and each lot was injected intracerebrally into a *Macacus rhesus*. None of the three monkeys of this series developed symptoms.

*Experiment H. Macacus rhesus 1.*—Jan. 5, 1914. 10 c.c. of filtrate of active K virus were injected intravenously. Six and twenty-four hours later 5 c.c. of blood were withdrawn, defibrinated, and injected separately into *Macacus rhesus* monkeys 2 and 3. The animal receiving the intravenous injection showed indefinite symptoms of excitability but developed no paralysis; it gradually became thinner and weaker and died on the twenty-first day after the injection. No apparent cause of death was found in the viscera. No lesions of poliomyelitis existed.

*Monkey 2.*—Jan 5. Received the 5 hour specimen of blood intracerebrally. Jan. 14. Excitable; arms paralyzed. Jan. 15. Legs paralyzed; prostrate. Jan. 17. Dead. Lesions of poliomyelitis present.

*Monkey 3.*—Jan. 6. Received the 24 hour specimen of blood intracerebrally. Jan. 14. Excitable; left arm weak. Jan. 15. Arms, legs, and back weak or paralyzed. Jan. 18. Prostrate. Jan. 24. Prostrate, moribund. Death hastened by ether. Lesions of poliomyelitis were present.

Experiments G and H bring out clearly the uncertainty of the intravenous mode of inoculation first in causing paralysis and second in maintaining the infectivity of the blood. It would appear that only when the blood is overwhelmed by the virus is it certainly infectious over a period of three days or less. Moreover, the failure

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of an intravenous dose of ten cubic centimeters of filtrate of K virus to cause paralysis when 0.2 of a cubic centimeter or less is a certainly effective dose by intracerebral injection, not only emphasizes the relative susceptibilities of monkeys to the two modes of inoculation but indicates also the possession of mechanisms by the body capable of excluding the virus within the blood from the nervous tissues. Whether it is the choroid plexus that is responsible or some other structure can only be surmised. It is conceivable that access of the virus to the central nervous system is secured only by way of the cerebrospinal fluid, in which case the virus within the blood must first penetrate the barrier of the choroid plexus which possibly takes place only when overwhelming doses are injected intravenously. When the virus is successfully inoculated subcutaneously or intraperitoneally it is always possible that the penetration to the central nervous organs is by way of the nerves. Hence the results may bear on the mode of infection of poliomyelitis in man.<sup>10</sup> In this connection it may be stated that the incubation period of the disease is longer with intravenous than with intracerebral inoculation. With the latter the average in a series of ten animals was 6.6 days and with the former in a series of six animals it was 10 days.

That the degree of virulence of the strain has a share in the effects is clearly illustrated by an experiment carried out with M A virus at a time when its infectious power had diminished.

*Experiment I.*—Oct. 3, 1913. 100 c.c. of filtrate of M A virus were infused into the leg vein of a *Macacus rhesus*. One hour and twenty-four hours later blood was withdrawn from the opposite leg and defibrinated. 3 c.c. were inoculated intracerebrally into each of two *Macacus rhesus* monkeys. None of the monkeys inoculated showed symptoms.

This experiment may have a bearing upon the unsuccessful inoculations with blood from human cases. There is little doubt of the presence of the virus in the one hour and the twenty-four hour samples of blood; and yet the quality of the virus was such that it failed to cause infection even when employed for intracerebral inoculation.

<sup>10</sup> Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

EXPERIMENTS WITH *STOMOXYS CALCITRANS*.

To the data on the infectivity of the blood given in the preceding pages there may be added briefly the results of a few experiments carried out with *Stomoxys calcitrans*. Rosenau and Brues<sup>11</sup> reported several instances of successful transmission of experimental poliomyelitis by means of the biting stable fly, a fact that was at first quickly confirmed by Anderson and Frost.<sup>12</sup> Through the courtesy of Dr. Rosenau one of us (Clark) was enabled to study his method of experimentation so that in the tests made by us Rosenau's method could be followed. Highly active M A virus was used in the experiments.

*Experiment 1.*—Oct. 1, 1912. 2 c.c. of a suspension of the spinal cord were inoculated intracerebrally into a *Macacus rhesus*. Oct. 4. Excitable. Oct. 6. Partial paralysis. Oct. 7. Prostrate. Oct. 9. Death.

On Oct. 4 and each day thereafter until Oct. 9 about 200 *Stomoxys calcitrans* were permitted to feed on the inoculated monkeys for two or three hours daily. The stable flies were caught in stables in New York City, and the losses from death were made up by adding fresh flies from time to time. The total number of flies employed in the experiment was about 400.

Oct. 7. A *Macacus rhesus* was inoculated intracerebrally with M A virus. Paralysis occurred on Oct. 16, prostration on the 17th, and death on the 18th. The same flies that fed on the preceding monkey were allowed to feed on this animal on Oct. 12 and again on Oct. 14, 15, 16, and 17. These flies were, therefore, permitted to feed ten times upon infected monkeys within fourteen days, five of the feedings taking place before, and five after the onset of paralysis.

From Oct. 6 to 18, except on three days, the 12th, 15th, and 16th, when fresh flies were introduced into the cage and fed first on infected animals, all these flies were given access, for two to three hours at a time, to four healthy monkeys (two *Macacus rhesus* and two *Macacus cynomolgus*). The healthy monkeys were therefore exposed to the flies ten times, covering a period of thirteen days. None of the monkeys developed symptoms of poliomyelitis.

At the conclusion of the feedings the dead flies were collected, ground up, and converted into a Berkefeld filtrate which was inoculated intracerebrally into a *Macacus rhesus* with negative result.

*Experiment 2.*—Six *Macacus rhesus* monkeys were inoculated intracerebrally between Oct. 18 and Nov. 9, 1912, with M A virus, and a *Macacus cynomolgus* with tonsils from a fatal human case of poliomyelitis. The rhesus monkeys became paralyzed and succumbed; the cynomolgus developed a partial paralysis and was etherized.

*Stomoxys* were allowed to feed on at least one of the monkeys of this series twenty-one times between Oct. 22 to Nov. 17, or a period of twenty-seven days.

<sup>11</sup> Rosenau, M. J., and Brues, C. T., Fifteenth International Congress of Hygiene and Demography, Washington, 1912.

<sup>12</sup> Anderson, J. F., and Frost, W. H., *Public Health Reports*, 1912, xxvii, 1733.

Monkeys in the preparalytic and paralytic stages were exposed to the flies. Since the death losses were large, about 1,400 flies in all were used in the experiment.

Beginning on Oct. 25 two healthy animals, one *Macacus rhesus* and one *Macacus cynomolgus*, were exposed to the bites of this lot of flies, for two to three hours at each exposure, on twenty-one days. The rhesus monkey appeared somewhat excitable on Nov. 4, and the excitability persisted until Nov. 11, when an examination of the cerebrospinal fluid showed it to be normal. No paralysis developed. The cynomolgus became somewhat excitable and developed weakness of one arm on Nov. 1. The condition did not progress, and on Nov. 4 the animal was etherized. Reinoculation of the spinal cord gave a negative result, and microscopical examination of the spinal cord and intervertebral ganglia showed them to be normal. Possibly the arm may have been hurt by the handling of the animal. This cynomolgus was replaced by another which was exposed to the flies from Nov. 4 to 17. It remained well.

At the conclusion of the experiment a filtrate was prepared from the dead flies, which was without effect when inoculated intracerebrally into a *Macacus rhesus*.

*Experiment 3.*—About 100 Stomoxys were allowed to feed on a *Macacus rhesus* during the two days following injection of a suspension of M A virus (spinal cord), and 150 Stomoxys were permitted to feed on a paralyzed and moribund monkey. The two lots were ground together and the filtrate from this emulsion was inoculated intracerebrally and intraperitoneally into a *Macacus rhesus*. The animal remained well.

The experiments conducted with Stomoxys gave negative results. In view of the tests on the infectivity of the blood given in the first part of this paper they are quite comprehensible, since it is only under exceptional circumstances that in intracerebrally inoculated monkeys the virus can be demonstrated in the blood by inoculation tests. The negative character of the tests with Berkefeld filtrates prepared from the bodies of the dead flies derives some significance from the single and exceptional successful inoculation with a similar filtrate prepared from *Cimex lectularius* (the bedbug), as reported by Howard and Clark.<sup>13</sup> Finally, it should be stated that a second series of experiments with the stable fly conducted by Anderson and Frost<sup>14</sup> was wholly negative, as were the comprehensive and critical experiments carried out by Sawyer and Herms.<sup>15</sup>

<sup>13</sup> Howard, C. W., and Clark, P. F., *Jour. Exper. Med.*, 1912, xvi, 850.

<sup>14</sup> Anderson, J. F., and Frost, W. H., *Public Health Reports*, 1913, xxviii, 833.

<sup>15</sup> Sawyer, W. A., and Herms, W. B., *Jour. Am. Med. Assn.*, 1913, lxi, 461.

SUMMARY.

Specimens of human blood taken during the paralytic stage of poliomyelitis and post mortem have proved not to be capable of infecting Macacus monkeys.

Specimens of monkey blood taken at various stages of experimental poliomyelitis have not proved as a rule to be capable of infecting monkeys. In a single instance, among ten tests, infection was secured with a specimen of blood removed at the beginning of the paralysis on the seventh day following an intracerebral inoculation.

When suspensions of the spinal cord from a paralyzed monkey have been injected into the brain or simultaneously into the brain and spinal canal, the blood removed from one to forty-eight hours later failed to cause paralysis after intracerebral injection.

When large volumes of active filtrate are injected into the circulation the blood remains infective for seventy-two hours at least, but may be no longer infective after ten days when the paralytic symptoms first appear. When, however, the filtrate is injected in smaller amount or when a filtrate of a less active virus is employed in large quantity, the blood either fails to convey infection or conveys it irregularly.

It is only when overwhelming quantities of an active virus are injected into the blood that paralysis results. The injection of moderate doses is not followed by paralysis, although the virus may still be detected in a blood sample twenty-four hours after the injection. The existence of a mechanism capable of excluding the virus within the blood from the central nervous organs is therefore inferred.

Infection is accomplished far less readily through the circulation than by means of the more direct lymphatic and nervous channels of communication with the central nervous system.

Several series of feeding experiments conducted with the biting stable fly (*Stomoxys calcitrans*) resulted negatively.

## THE RELATION BETWEEN THE FAT CONTENT OF THE BILE AND FATTY CHANGES IN THE LIVER.\*

By EDWIN R. LE COUNT, M.D., AND ESMOND R. LONG.

(From the Pathological Laboratories of Rush Medical College and the University of Chicago, Chicago.)

Although numerous analyses of bile are reported in the literature, relatively few deal with the chemistry of the bile in the pathological state, and little systematic investigation seems to have been made of the bile in diseased conditions of the liver. A few observations are recorded, most of them old, which seem to show that, in a general way, acute inflammatory conditions of the liver lead to an increased secretion of the bile constituents, although the amount of water eliminated may be small, while atrophy and chronic degenerations result in a much lowered secretion.

Concerning the composition of the bile secreted by fatty livers, we have found only a few observations. von Gorup-Besanez<sup>1</sup> saw fat droplets and palmitin crystals in the bile in cases of typhoid fever and tuberculosis. Ritter,<sup>2</sup> in two publications on the composition of colorless bile, found a normal, unvarying fat content of the gall bladder bile in what were diagnosed macroscopically as "fattily degenerated" livers, three in man and one in a dog. However, as the livers were not reported as being excessively fatty, that is, fattily infiltrated, and as no analysis for fat was made, the diagnosis being macroscopic only, and as the determination of fat and cholesterol only without consideration of the fatty acid content of the soaps of the bile, seems to have been made, as far as we can tell from the published figures, it is doubtful if the results are of much value as an indication of the general condition of the bile in fatty changes in the liver. Accordingly it seems that Hoppe-

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<sup>1</sup> von Gorup-Besanez, E. F., Lehrbuch der Chemie, 3d edition, Braunschweig, 1874, iii, 525.

<sup>2</sup> Ritter, E., *Jour. de l'anat. et de la physiol.*, 1872, viii, 181; *Compt. rend. Acad. d. sc.*, 1872, lxxiv, 813.

Seyler's<sup>3</sup> quotation of Ritter's results as settling the question of the composition of bile secreted by fatty livers is unwarranted. Hoppe-Seyler states in the same passage that the bile of geese may remain normal in fat composition in high degrees of fatty infiltration of the liver. On the other hand, more recently, Gilbert and Jomier<sup>4</sup> have published the results of investigations in which an excretion of considerable amounts of fat in the bile was observed histologically in experimental fatty infiltration of the liver in rabbits, following the ingestion of meals rich in fat.

In our investigation we have obtained livers of varying degrees of fatty change and the corresponding gall bladder bile from a number of cases, shortly after death, and subjected both to analysis for fat,—the liver by extraction with hot alcohol and ether, and the bile by the Kumagawa-Suto method for fat estimation, in which the total fatty acid and cholesterol content is determined after saponification. This method gives lower results for the lipoid content of the bile than those usually reported in the literature, partly because of the loss of the glycerol radicle of the fats and the non-lipoidal elements of lecithin in the saponification, but largely, probably, because of the elimination of certain non-lipoidal constituents of the bile, such as cholic acid and similar substances, which are soluble in ordinary diethyl ether, but insoluble in petroleum ether, the final solvent in the Kumagawa method.

It must be admitted, of course, in view of our imperfect acquaintance with the conditions of the bile in the gall bladder, the amount of reabsorption, and so forth, that a study of the bile found in the gall bladder does not furnish a perfect indication of the condition of the bile as secreted originally by the liver. We know that in the biliary tract the bile becomes concentrated, while some substances, notably mucin, are added, and the question arises whether some fat may not be added and also if some fat may not be lost. In this connection we note that Virchow<sup>5</sup> found that a reabsorption of bile fat takes place from the gall bladder epithelium. He states further that in the fattiest livers he has found no fatty change in

<sup>3</sup> Hoppe-Seyler, F., *Physiologische Chemie*, Berlin, 1881, 317.

<sup>4</sup> Gilbert A., and Jomier, J., *Arch. de méd. expér. et d'anat. path.*, 1905, xvii, 1.

<sup>5</sup> Virchow, R., *Virchows Arch. f. path. Anat.*, 1857, xi, 574.

the epithelium of the gall bladder. On the other hand, we ourselves have several times observed in dogs fatty changes in the gall bladder epithelium when the liver itself was not fatty. In spite of these facts, which make it unsafe to attempt to lay down general principles on the basis of evidence from analyses of gall bladder bile, we felt that a study of the liver and gall bladder bile in man, in various degrees of fatty change of the liver, would be of considerable interest, and accordingly undertook the investigation with the expectation of following it up later with experimental studies upon dogs with biliary fistulæ.

In text-figures 1 and 2 and in table I the results are summarized.

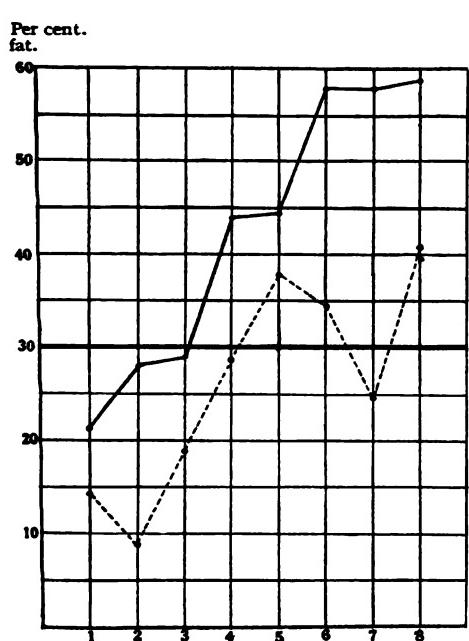
TABLE I.

Case No.	Liver.			Bile.			Remarks.
	Ratio of total fat to total dry weight.	Ratio of lecithin to fat-free tissue.	Ratio of cholesterol to fat-free tissue.	Amount m.	Total fat in bile per thousand.	Cholesterol in bile per thousand.	
1	21.50%	1.05%	1.61%	30	8.6	1.1	Skull fracture and external violence.
2	27.60%	1.43%	2.18%	30	5.0	0.6	Wood alcohol poisoning.
3	28.78%	1.23%	2.86%	15	11.3	2.0	Lye poisoning and bronchopneumonia.
4	44.10%	1.40%	3.20%	15	17.5	1.3	Pulmonary tuberculosis.
5	44.64%	1.23%	2.68%	15	22.8	1.6	Gun shot wound of head. Liver fatty.
6	57.80%	1.49%	3.41%	14	20.4	4.0	Chronic alcoholism.
7	57.80%	—	—	64	14.9	2.3	Chronic morphinism(?)
8	58.66%	1.25%	3.59%	40	24.2	4.0	Alcoholism and chronic nephritis.

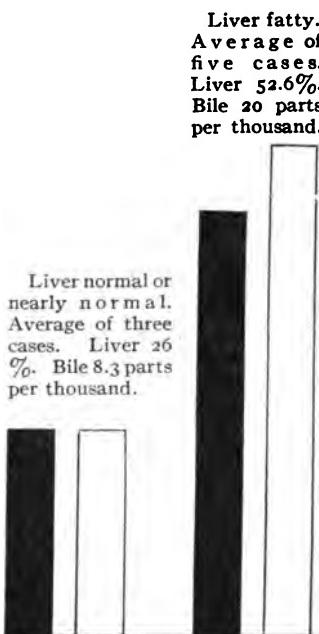
In cases 1, 2, and 3 the liver showed little or no fatty change by analysis or inspection. Cases 4, 5, 6, 7, and 8 were fatty livers. The 30 per cent. line in figure 1 separates the two types. The total fat and lipoid content of the eight livers examined is represented on the solid line in the chart in order of increase, while the fat (fatty acid plus cholesterol) content of the corresponding gall bladder bile is represented on the same ordinate in each case, on a scale convenient for comparison with the liver curve, the broken line constituting the bile curve. As will be noted, the bile curve

is irregular in character and does not correspond exactly with the liver curve, but the chart shows that on the whole the gall bladder bile of the fatty livers averages higher in fat than that found in the gall bladders of the livers which were not fatty.

This fact is illustrated more strikingly by text-figure 2. The normal or nearly normal livers average 26 per cent. fat, and the



TEXT-FIG. 1.—Liver. One space on the ordinate = 5 per cent. of dry weight soluble in ether. .... Bile. One space = 3 parts of fatty acid + cholesterol per 1,000 parts of bile.



TEXT-FIG. 2. Comparison of fat content of liver and corresponding gall bladder bile of livers represented above the heavy line with those represented below the line in text-figure 1. Fat content of liver expressed in per cent. of dry weight soluble in ether. Fat content of bile expressed in parts of bile per 1,000.

corresponding gall bladder biles 8.3 parts per thousand. The figures are represented by columns of equal height, and columns representing the average fat content of the fatty livers and their gall bladder biles are placed beside them for comparison, the second

liver column, representing an average fat content of 52.6 per cent of the dry weight, being drawn to the same scale as the first liver column, and the second bile column, representing an average of 20 parts per thousand, being drawn to the same scale as the first bile column. A striking parallelism is thus seen, which seems to indicate that in general the fat content of the bile secreted by fatty livers is greater than that of the bile from normal livers, in spite of the possible objections noted above, in view of which, moreover, an absolute parallelism could not be expected. In table I the figures for the analyses and the causes of death are given for each case.

It was thought of interest to determine the lecithin and cholesterol. The former was estimated by the method of Koch and Woods,<sup>6</sup> and the latter by the method of Corper,<sup>7</sup> which was found to yield excellent results and closely agreeing duplicates. As is usually found, the ratio of the lecithin to the dry, fat-free tissue is nearly a constant one, lecithin making up a fairly definite proportion of the cell protoplasm, in both normal and fatty livers, the increased fat of the latter being chiefly infiltrated body fat. A similar relation might be expected to hold for the cholesterol, but our figures show an increase in the amount of this substance accompanying the infiltration of adipose tissue fat.

We wish to express our indebtedness to Dr. E. J. Witzemann for the determination of the dry weight of the liver samples, which was always made immediately after the autopsy.

<sup>6</sup> Koch, W., and Woods, H. S., *Jour. Biol. Chem.*, 1905-06, i, 203.

<sup>7</sup> Corper, H. J., *Jour. Biol. Chem.*, 1912, xii, 197.

## SOAP AS FERMENT-INHIBITING AGENTS.

### STUDIES ON FERMENT ACTION. X.\*

By JAMES W. JOBLING, M.D., AND WILLIAM PETERSEN, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons,  
Columbia University, New York.)

The use of the antitryptic index of the blood as a clinical test, particularly in cancer, and the pathological conditions in which ferment action appears to be held in abeyance, especially in caseation in tuberculosis and syphilis, in anemic infarcts, and in the exudate in lobar pneumonia, make the study of ferment-inhibiting substances of great importance. Many substances are considered to have the property of inhibiting the activity of enzymes, but in this paper we shall refer chiefly to the proteolytic enzymes acting in an alkaline medium.

Trypsin and leucoprotease lose their activity when heated at temperatures above 60° C., though according to Salkowski (1) trypsin when dry can be heated to 160° C. without being destroyed. Strong acids and alkalies quickly destroy trypsin, but Chittenden and Cummins (2) found it much more resistant to these agents in the presence of protein. Kudo (3) states that all the mineral acids are active in this respect, sulphuric acid being active in a dilution of one in a thousand, while the organic acids are much less so. Weiss (4) reports that while 0.05 per cent. sodium chloride increases the activity of trypsin, 10 per cent. decreases it. Most of the writers who have investigated the subject find that chloroform, toluol, and thymol inhibit the action of trypsin to a slight degree, but the necessity of a preservative in their experiments caused most workers to ignore this slight inhibition. Bayliss (5) and Abderhalden and Gigon (6) found that some of the products of tryptic activity possess the property of inhibiting the action of the ferment, the free amino acids being more active than the polypeptides. It has been known for several years that blood serum is capable of inhibiting the proteolytic power of trypsin, but in spite of the work of Camus and Gley (7), Charrin and Levaditi (8), Landsteiner (9), and others, we are still ignorant concerning its nature and significance.

In the course of our studies on ferment action we observed that soaps of certain fatty acids were capable of inhibiting the action of trypsin and leucoprotease. This observation seemed to be of importance, as soaps of a similar nature are present in the body, and the possibility of their acting in the same manner

\* Received for publication, December 23, 1913.

caused us to make a more careful study of the general phenomena relating to the inhibition of ferment activity by these substances.

Neumann (10), while studying the influence of a fat-rich diet on the digestion of infants, observed that sodium oleate inhibited the action of trypsin. For most of his work he used the Fuld-Gross technique. In some of our preliminary experiments we used the same technique, but soon discarded it in favor of the one which will be described later. According to the Fuld-Gross method the mixture of ferment and substrate is incubated for two hours and then made slightly acid by adding a few drops of a solution containing 5 per cent. acetic acid in 50 per cent. alcohol. The amount of digestion is determined by the degree of cloudiness, the more complete the digestion the clearer the contents of the tube after acidifying. Subsequent experience convinced us of the unreliability of this method, particularly when dealing with lipoidal substances. The acidifying not only coagulates the undigested protein, but also causes the lipoidal substances, particularly the fatty acids, to separate as flocculi, a condition which makes it impossible to determine the extent of proteolysis. Neumann added an excess of alcohol to cause a solution of the precipitated acids and thus solved one difficulty; but he added another by diluting the protein to such a degree that it became a problem to determine the extent of proteolysis.

Two ferments, trypsin and leucoprotease, were used in our work. The trypsin was obtained by extracting commercial pancreatin with N/50 sodium carbonate. After extraction for twenty-four hours, the solution was filtered, and the trypsin in the filtrate precipitated by the addition of nine volumes of alcohol to which sufficient acetic acid had been added to make the whole mixture slightly acid to litmus. This extraction and precipitation was repeated three times. The final product was much stronger than the original and the amount needed to cause complete digestion in the control tubes did not contain sufficient nitrogen to give a definite color when Nesslerized. The leucoprotease was prepared from human pus in the same manner, but the amount required to cause complete digestion in the control tubes contained more nitrogen than the concentrated trypsin. In each experiment the amount of nitrogen was determined in the control tubes and proper correction made in calculating the amount of total incoagulable nitrogen.

Edestin was used as a substrate for several experiments, but later it was discontinued in favor of a 1 per cent. casein solution, the latter being much more soluble in an alkaline medium, and in addition it was noted in precipitating the coagulable protein that a slight excess of acid caused the edestin precipitate to redissolve. After being incubated for the necessary time, the contents of the tubes

were acidified with a mixture containing 10 per cent. glacial acetic acid and 20 per cent. sodium chloride, and the tubes placed in boiling water for five to ten minutes. The mixtures were then filtered through kaolin and the incoagulable nitrogen was determined by the method recommended by Folin (11). The amount of nitrogen found in the control tubes was subtracted from each of the others and the results were given in percentages of total digestion.

The time allowed for incubation was not the same in all experiments. In our preliminary work we found that the concentrated trypsin, prepared as described above, deteriorated rapidly when once dissolved. For this reason the trypsin solution was prepared immediately before being used, one cubic centimeter representing 0.002 of a gram of trypsin. Several tubes containing only casein and trypsin were placed in the incubator at the same time as the tubes or flasks containing the mixtures of soaps. One of the tubes containing casein and trypsin was removed after one hour and tested. After that the other tubes were removed at intervals of fifteen minutes, also tested, and as soon as digestion was found to be complete the remainder were immediately removed, acidified, and boiled.

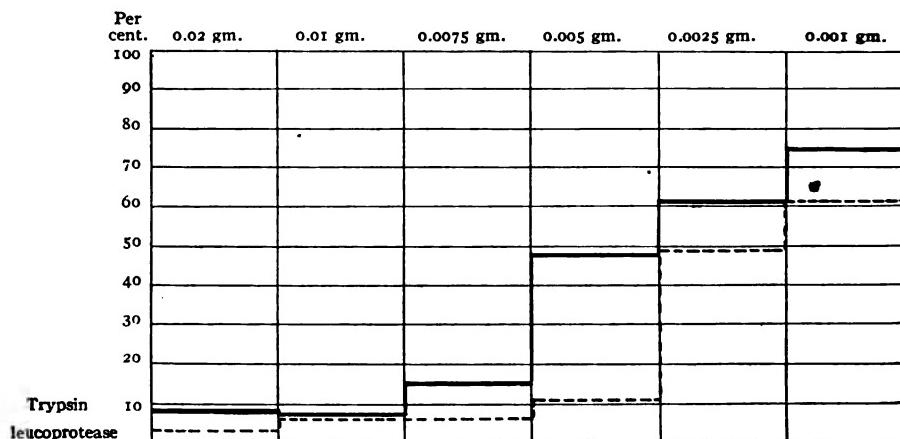
The protocols are not given for each experiment as the charts present the results more clearly. Table I gives the number of controls used in each experiment, and also the amount of nitrogen obtained by complete digestion and as a result of the inhibiting action of the soaps.

In most instances we did not know the identity of the acids used in the preparation of the soaps, but the isolation and identification of these with further work on their ferment-inhibiting action is now under way. The three supposedly pure soaps were Kahlbaum's preparations, and consisted of sodium oleate, sodium palmitate, and sodium stearate. On examination we found that the sodium oleate had become saturated, making it useless as an oleate, though it was used in several experiments as a representative of the saturated fatty acids.

The majority of the soaps were obtained by saponifying linseed, olive, cod-liver, hempseed, and castor oils, as these oils are known to contain a large number of unsaturated fatty acids. The oils were saponified with alcoholic potash, and the soaps extracted re-

peatedly with petroleum ether. The acids were then liberated by hydrochloric acid, taken up in ether, the ether evaporated, and the acids again saponified. This process was repeated and the acids were preserved pure until needed. The soaps used in the work were rarely more than two or three days old.

Later when it was found that the unsaturated fatty acids were the active inhibiting agents, the lead soap-ether method of obtaining these in higher concentrations was used. The separation of the saturated and unsaturated fatty acids by this method is not complete, but is sufficiently so to enable us to show marked difference between the action of the ether-soluble and insoluble fractions. I

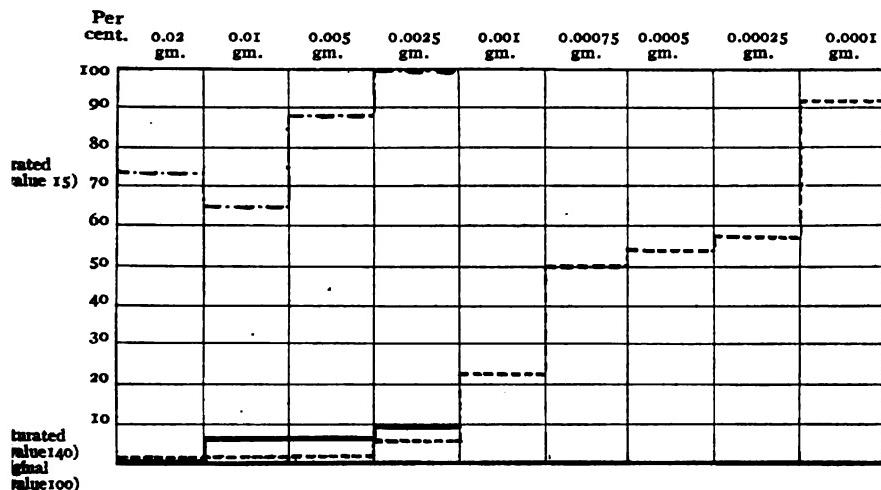


TEXT-FIG. 1. Effect of sodium oleate on tryptic and leucoproteolytic digestion.

nearly every instance the iodin value of the preparations was determined before they were used.

One of the first soaps investigated was that prepared from olive oil. Its influence on leucoprotease and trypsin is shown in text-figure 1. In this experiment the soap and ferment were mixed, incubated for thirty minutes at 37° C., and the casein was then added. This procedure was adopted in all the subsequent experiments. Text-figure 1 shows that even 0.001 of a gram of the soap is sufficient to affect materially the activity of both ferments. In this and in subsequent charts the control tubes showing 100 per cent. of digestion are not given.

Efforts were then made to determine if the inhibiting action displayed by these soaps was due to the saturated or unsaturated fatty acids present. Soaps from nearly all the oils were tested in a similar manner, but as uniform results were obtained, we shall give only one example. Text-figure 2 shows the results obtained with



TEXT-FIG. 2. Effect of saturated and unsaturated linseed soaps on tryptic digestion.

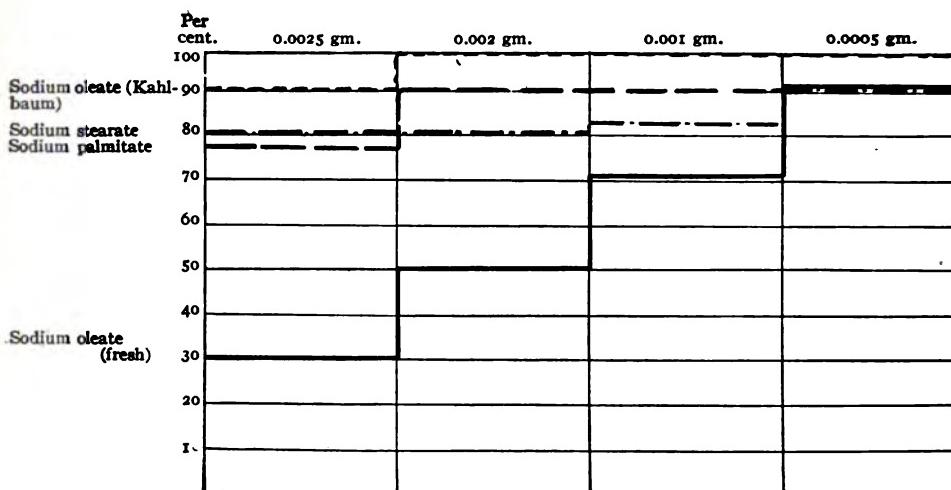
the soap prepared from linseed oil. The ether-lead soap method was used in separating the groups of acids. The chart indicates that the unsaturated fatty acids are the active inhibiting agents. It is well known that the ether-lead soap method does not give a complete separation of the saturated and unsaturated fatty acids, and this probably explains why the saturated fraction, the ether-insoluble lead soaps, still retained about one fourth of its inhibiting action, whereas the unsaturated fraction, the ether-soluble lead soap, now caused complete inhibition.

During the progress of the work we obtained three of Kahlbaum's supposedly pure soaps, sodium oleate, sodium palmitate, and sodium stearate. The results obtained with these three soaps and with one prepared by ourselves from olive oil are shown in text-figure 3. The iodin value of the linseed oil soap prepared by us was 90.

Our previous experiments indicated that the soaps of all the unsaturated fatty acids acted as inhibiting agents. We were therefore

surprised to note that the Kahlbaum sodium oleate was inactive. The solution of the problem was soon found, the sodium oleate had no iodin value; in other words, the oleic acid, through age or some other cause, had become saturated. The sodium soaps of palmitic and stearic acids, as we had anticipated, had no influence on either trypsin or leucoprotease.

All our experiments so far indicate that the inhibiting agents present in the soaps are the unsaturated fatty acids. The question therefore naturally presents itself: Can we by a saturation of the



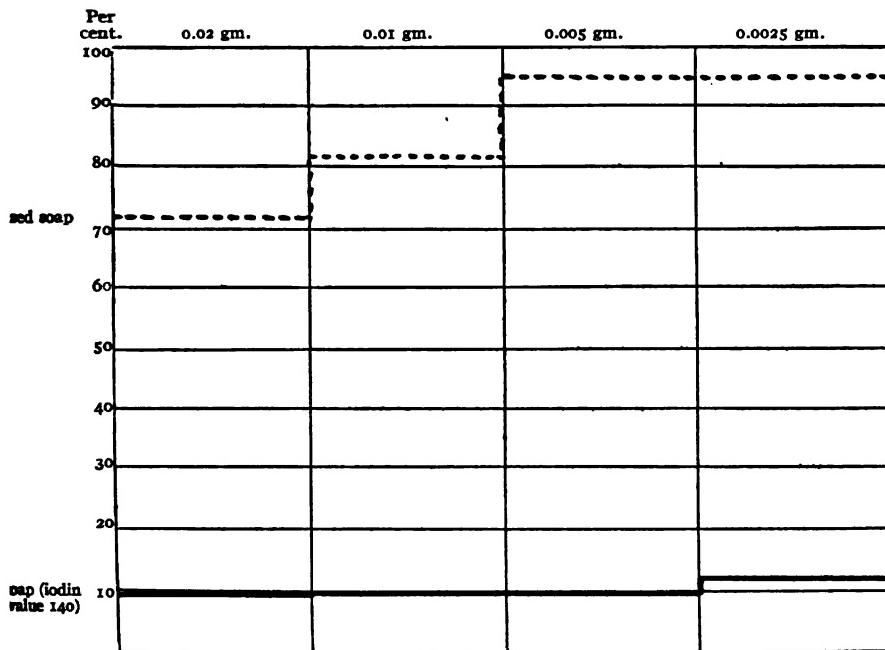
TEXT-FIG. 3. Effect of sodium oleate, sodium stearate, and sodium palmitate on tryptic digestion.

free bonds with iodin, for instance, destroy the inhibiting action and cause them to act like the soaps prepared with stearic, palmitic and other saturated acids?

The results of one of the experiments made to enlighten us on this point are given in text-figure 4. This experiment was conducted with the ether-soluble lead soap fraction. To five cubic centimeters of a 1 per cent. solution of the soap were added 0.05 of a gram of iodin and a few crystals of iodide of potassium, and the mixture was permitted to stand over night. The following morning it was shaken repeatedly with chloroform, until fresh portions of the

latter remained clear, showing that there was no more free iodin. Text-figure 4 shows the action of the unsaturated soap before and after it was treated with iodin. Repeated tests had already shown that the small amount of iodin which might still remain would have a slight inhibiting action. This would explain why the soap still had some inhibiting action, though it may also be due to the fact that the acids were not completely saturated. The results of saturating the free carbon bonds are so obvious as to call for no comment, and also explain why the commercial sodium oleate was inactive.

Similar experiments made with sodium soaps of unsaturated fatty acids obtained from other sources indicated that the inhibiting



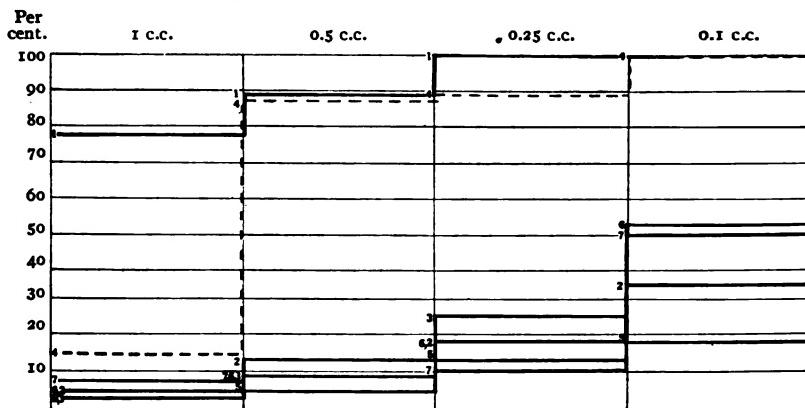
TEXT-FIG. 4. Effect of saturation of unsaturated linseed soaps with iodin.

action was due to the degree of unsaturation and that it could be almost entirely removed by saturation with iodin.

Our next experiments were made to determine whether the activity of the various soaps was dependent upon the number of unsatu-

rated bonds, as determined by their iodin values, or whether certain ones containing an equal number of unsaturated bonds were more active.

In this experiment soaps prepared from linseed oil, hempseed oil, castor oil, olive oil, croton oil, and cod-liver oil were used. After their respective iodin values had been determined, they were made up in such strengths that one cubic centimeter of each had the same iodin value; thus croton oil soap having less than half the value of the linseed oil soap was made up twice as strong. Text-figure 5



- |                             |                  |                         |
|-----------------------------|------------------|-------------------------|
| 1 = saturated linseed soap. | Iodin value 15.  | Solution 5.0 per cent.  |
| 2 = sodium oleate.          | Iodin value 53.  | Solution 1.9 per cent.  |
| 3 = croton soap.            | Iodin value 55.  | Solution 1.8 per cent.  |
| 4 = castor oil soap.        | Iodin value 60.  | Solution 1.7 per cent.  |
| 5 = hempseed soap.          | Iodin value 100. | Solution 1.0 per cent.  |
| 6 = cod-liver oil soap.     | Iodin value 90.  | Solution 1.1 per cent.  |
| 7 = linseed soap.           | Iodin value 135. | Solution 0.74 per cent. |

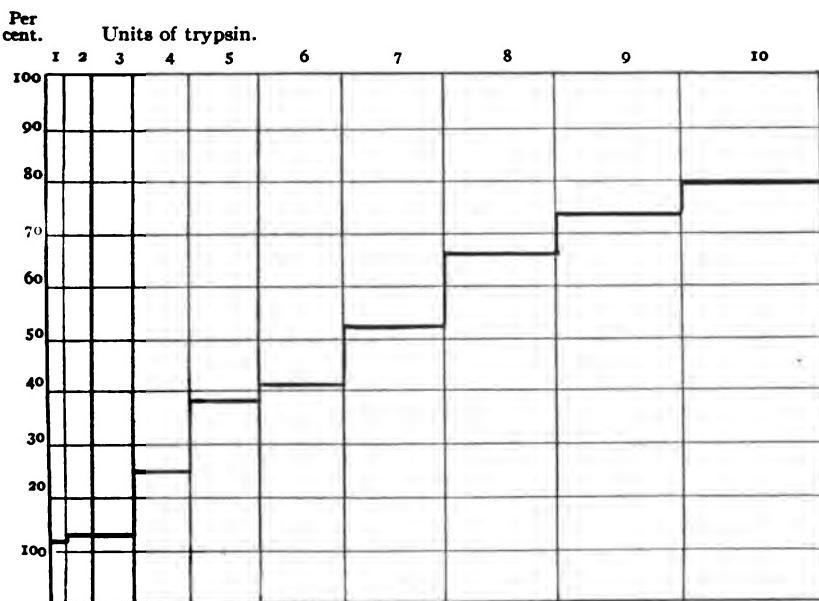
TEXT-FIG. 5. Relation of iodin value to inhibiting action of soaps on tryptophane activity.

gives the results of this experiment. The lines shown on the chart indicate, with one exception, that the activity of the soaps is in proportion to their iodin values.

Here we have the first indication that the unsaturated fatty acids are not equally active as inhibiting agents, for the soap prepared from castor oil appears to be considerably less active. The inhibition obtained with one cubic centimeter was almost as great as that noted with the other soaps, but subsequent dilution proved it to

much less active. With the exception of that prepared from linseed oil, the soaps used in this experiment were not obtained by the ether-lead soap method, and so their iodin values were low. It is possible that some of the unsaturated fatty acids are much more active than others in this respect. In fact, a part of our recent work indicates that those obtained from a certain source are much more active than any of those we have just discussed.

In the preceding experiments it has been demonstrated that 0.005 of a gram of the linseed oil soap was sufficient to prevent the action of the standard amount of trypsin. It was also found, in the standard proportions of ferment and substrate used, that the inhibiting action was still present in tubes containing much smaller amounts. In fact complete digestion was first obtained in the tube containing



TEXT-FIG. 6. Effect of increasing amounts of trypsin with single unit of soap.

0.0001 of a gram. In our next experiment we wished to find out if the smallest amount of soap producing nearly complete inhibition with the standard amounts of trypsin and casein would have any influence on larger amounts of the ferment. Text-figure 6 shows

the influence of the soap on increasing amounts of the ferment. 0.1 of a cubic centimeter of the ferment solution caused complete digestion in the control tube. The demonstration that such a small amount of soap, 0.005 of a gram, was able to affect materially the action of ten times the standard amount of ferment, indicates the great activity of these substances as inhibiting agents.

In the previous experiments we have shown that the unsaturated fatty acids inhibit proteolysis in an alkaline medium and that the degree of inhibition bears a close relation to their iodin values, but these experiments do not give us much information as to how the inhibition is effected. Is it due to a direct and permanent binding of the ferment with the unsaturated fatty acids, or to simple inhibition of ferment action, the ferment itself not being destroyed? If there is a true binding it would be difficult to extract the ferment after incubating with soap, but it might be done if the inhibition were due to some other cause.

Our next experiment was planned with the hope that some information might be obtained as to the nature of the inhibiting action. In this experiment the soaps of the saturated and unsaturated acids separated by the ether-lead soap method were mixed with the ferment and incubated for thirty minutes. The mixtures were then made slightly acid with hydrochloric acid and extracted with ether. After having removed the ether, the solution was made slightly alkaline, the casein added and again incubated. One control tube of ferment without soap was treated in the same manner so as to rule out the effect of the acid and ether. In this experiment we wished to find out if the soap simply inhibited the action of the ferment or destroyed it. Table I gives the results of this experiment.

TABLE I.

Trypsin.	1 per cent. un-saturated lin-seed soap, iodin value 130.	1 per cent. saturated lin-seed soap, iodin value 15.	Sodium chloride solution.	1 per cent. casein.	Total incoagulable nitrogen.	Digestion.
1 0	0	0	3.0 c.c.	2.0 c.c.	0.04 mg.	2 %
2 0.2 c.c.	0	0	2.8 c.c.	2.0 c.c.	1.66 mg.	100 %
3 0.2 c.c.	0.5 c.c.	0	2.3 c.c.	2.0 c.c.	0.10 mg.	6 %
4 0.2 c.c.	0	0.5 c.c.	2.3 c.c.	2.0 c.c.	1.30 mg.	80 %
5 0.2 c.c.	0	0	2.8 c.c.	2.0 c.c.	1.50 mg.	90 %
6 0.2 c.c.	0.5 c.c.	0	2.3 c.c.	2.0 c.c.	0.20 mg.	12 %
7 0.2 c.c.	0.5 c.c.	0.5 c.c.	2.3 c.c.	2.0 c.c.	1.66 mg.	100 %

The first four tubes in the table are the untreated controls. The first tube shows the percentage of incoagulable nitrogen in the casein alone; the second, the total nitrogen in complete digestion; the third and fourth show the influence of the saturated and unsaturated soaps on tryptic activity. Tubes 5, 6, and 7 show the influence of acid and ether extraction. Tube 5 contained no soap but was treated in the same way as Nos. 6 and 7 to make certain that the method used had not destroyed the ferment. Tube 6 containing the unsaturated soap shows that the ferment action is almost inhibited, while in tube 7 containing the saturated soap there is no inhibition. In other words, incubating the ferment with soaps of the unsaturated fatty acids for thirty minutes causes its destruction, the removal of the acids after incubation not being sufficient to reactivate the ferment, while the ferment present in the tube containing the saturated acid, though treated in the same manner, is not destroyed.

The results obtained in this preliminary study of the action of the soaps prepared from the unsaturated fatty acids suggest that they may play an important part in the body, particularly in certain pathological processes. Whether this inhibition of ferment action is due to a binding of the ferment with the unsaturated carbon atoms, or whether to some physical condition brought about by the presence of the soap, remains to be determined. If due to some physical condition brought about by the presence of the soap, similar results ought to be obtained with the saturated fatty acids, but we have demonstrated that the latter are inactive in this respect. In addition, the experiments conducted with soaps saturated with iodin show that the presence of unsaturated bonds are necessary for the development of this property. Further proof that this action is due to the degree of unsaturation is afforded by text-figure 5, which indicates that the activity of the soaps is proportionate to their iodin value, while the soaps having no iodin value, sodium stearate and sodium palmitate, are inactive.

#### SUMMARY.

1. Sodium soaps prepared from olive oil, croton oil, cod-liver oil, linseed oil, etc., have the property of inhibiting the action of trypsin and leucoprotease.

2. The activity of these soaps is dependent upon the degree of unsaturation of the fatty acids and is in proportion to their iodine value.

3. Saturation of the acids with a halogen (iodine) causes a loss of this property.

4. Soaps of the saturated fatty acids tested do not have this influence on fermentations.

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## FERMENT-INHIBITING SUBSTANCES IN TUBERCLE BACILLI.

### STUDIES ON FERMENT ACTION. XI.\*

By JAMES W. JOBLING, M.D., AND WILLIAM PETERSEN, M.D.

(*From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.*)

A great deal of work has been done in the attempt to explain the processes that lead to the production of caseation in tuberculosis. By some it has been ascribed to anemia, the tubercle being avascular, and by others to the action of toxins derived from the tubercle bacilli.

Auclair (1) believes that the caseation is due to specific toxins of the nature of fat, which are soluble in ether, chloroform, benzine, and xylol. He treated tubercle bacilli with one or more of the above agents, evaporated the solvents, and suspended the extracted substances in water. These emulsions, when injected into the subcutaneous tissues of animals, produced typical caseous abscesses. When injected into the trachea of guinea pigs, caseous areas appeared in the lungs.

Schmoll (2) analyzed caseous material and found it almost free from proteoses, which indicates that autolysis is slight; but he does not explain the lack of autolysis. The work of Schmoll has been confirmed by others, and we have obtained similar results.

Anemia due to the occlusion of the blood vessels may be an important factor in causing caseation in the chronic forms of tuberculosis where the areas are surrounded by a layer of connective tissue. However, in acute caseous pneumonia in which there is a catarrhal exudate rapidly becoming caseous, some other explanation appears to be necessary. In such an exudate there are numerous cells known to contain ferments, yet these die and disintegrate, and, as far as can be determined, the ferments remain inactive. We must therefore look for some other factor to explain the process of caseation, and this we believe we have found in the tubercle bacillus, a ferment-inhibiting substance. We observed two years ago that the

\* Received for publication, January 5, 1914.

antitryptic action of sera was lost or greatly decreased when the sera were preserved with chloroform. This observation suggested to us the possibility that the antiferment properties of the blood were due to substances of the nature of lipoids, and the lack of autolysis in caseation and in anemic infarcts to substances of similar nature.

With this hypothesis we decided to study the influence of the ether-soluble substances of tubercle bacilli on proteolysis. It has been shown by numerous investigators that the ether-soluble substances constitute 25 to 35 per cent. by weight of the bacilli, and of these extracts about 17 per cent. are composed of fatty acids. Both human and bovine tubercle bacilli were used in the work.<sup>1</sup>

Our first experiments were conducted with ether- and alcohol-soluble substances obtained from the tubercle bacilli. After the solvent had been evaporated, the material remaining was taken up in methyl or ethyl alcohol and suspended in a 0.9 per cent. solution of sodium chloride. In some experiments the emulsion was used just as prepared; in others the alcohol was first evaporated at low temperatures. The emulsion, in various dilutions, was then mixed with trypsin, incubated for thirty minutes, and the casein added. As far as we could determine there was no inhibition of ferment action.

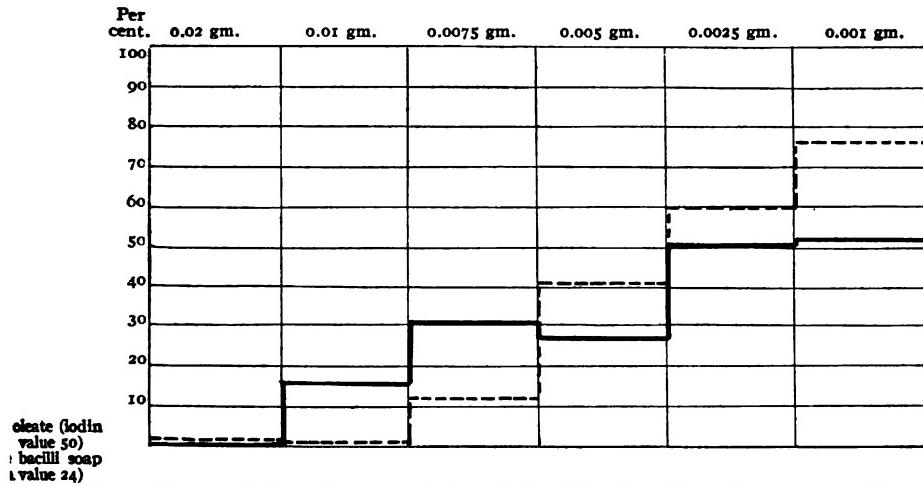
In the preliminary experiments we used the Fuld-Gross technique but, for reasons given in our preceding paper (3), we soon discarded it in favor of the following. After incubating the mixtures for a certain period of time, they were acidified with a solution containing 10 per cent. acetic acid and 20 per cent. sodium chloride. The tubes were then placed in boiling water for five to ten minutes, filtered through kaolin to remove the coagulated protein, and the non-coagulable nitrogen was determined according to the method recommended by Folin (4).

This experiment failed to demonstrate the presence of an inhibiting agent. This may have been due to the agent being in an emulsion and not a solution, thus preventing its coming into intimate contact.

<sup>1</sup> We wish to thank Dr. Hitchens, of Glenolden, Pa., and Dr. Grund, of the New York Board of Health, for supplying us with large amounts of human and bovine tubercle bacilli.

tact with the ferment. We then fractionated the extracted material so that at least a part, the fatty acids, might be prepared in such a manner that they could be brought into solution. The material was dissolved in ether and precipitated with acetone. The acetone fraction was evaporated to dryness on the water bath and the resulting substance saponified with alcoholic potash. The soap was dissolved in water and repeatedly extracted with petroleum ether in order to remove any unsaponifiable matter. The acids were then liberated with hydrochloric acid, taken up with ether, washed with water, and resaponified.

The ferments were prepared and standardized according to the

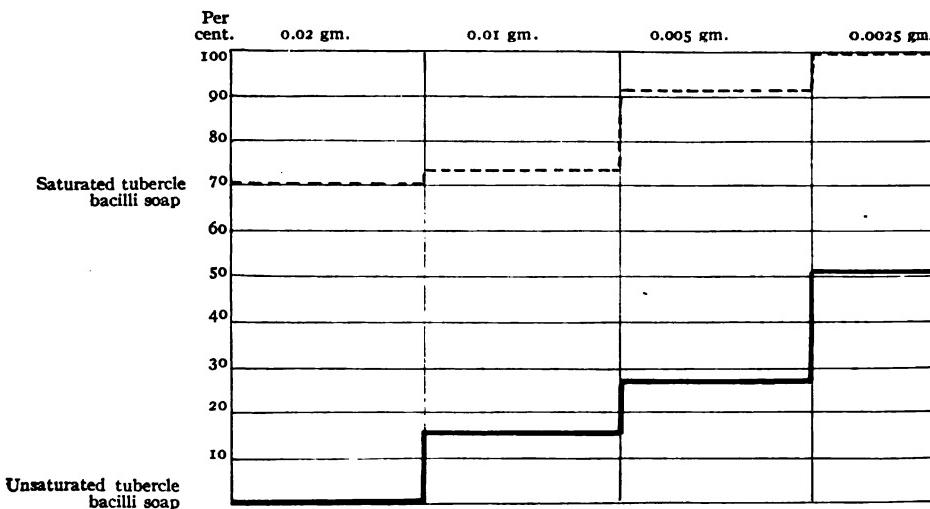


TEXT-FIG. I. Effect of soaps of tubercle bacilli and sodium oleate on tryptic digestion.

method described in our previous paper (5). The soaps, in 1 per cent. solutions, were mixed with trypsin in varying amounts, incubated for thirty minutes, and the casein was then added. After the final incubation of two hours, the coagulable protein was removed and the incoagulable nitrogen determined. Text-figure I shows the results obtained with the soap prepared from extractives of the tubercle bacilli. A soap prepared from olive oil was used as a control. The dotted and heavy black lines indicate the percentage of digestion present in the tubes containing soaps as compared with the controls containing trypsin and casein alone.

The results of this experiment show that in the final dilution the soap prepared from tubercle bacilli is more active as an inhibiting agent than the one prepared from olive oil. The iodin value of the soap prepared from the tubercle bacilli was 24, of the olive oil soap 50. The relation that the iodin value of the soaps bears to their activity as inhibiting agents was discussed in our previous report. It will be dealt with here in relation to the one prepared from tubercle bacilli.

In the previous paper (5) on the influence of soaps of the fatty acids on inhibition of ferment action, we demonstrated that the unsaturated fatty acids were the active agents, while the saturated fatty acids exerted but little if any inhibiting action. We were unable to obtain a complete separation of the saturated from the unsaturated acids and so could not remove entirely the inhibiting



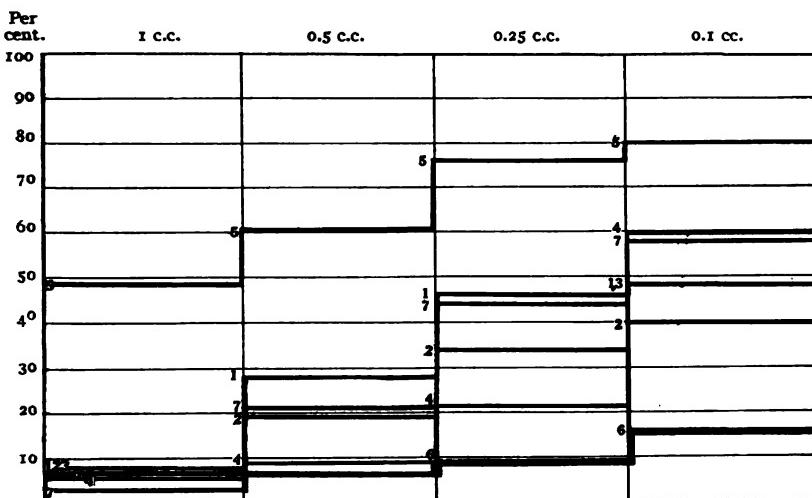
TEXT-FIG. 2. Effect of saturated and unsaturated soaps of tubercle bacilli on tryptic digestion.

action, but the differences in their activity following their partial separation by the ether-lead soap method were so striking as to be conclusive. The soaps of the saturated fatty acids tested, sodium stearate and sodium palmitate, were inactive.

We undertook to determine if the same is true of the soaps pre-

pared from tubercle bacilli. Lead soaps were prepared according to the usual method. The acids were set free with hydrochloric acid, washed, resaponified with sodium alcoholate, and evaporated to dryness. Text-figure 2 gives the results obtained with soaps prepared from saturated and unsaturated fatty acids.

The results obtained with the ether-soluble and insoluble fractions are as conclusive as those described in the preceding report for other soaps. The ether-lead soap method does not give a complete separation of the saturated and unsaturated fatty acids, but the separation is sufficient to demonstrate which is the active fraction.



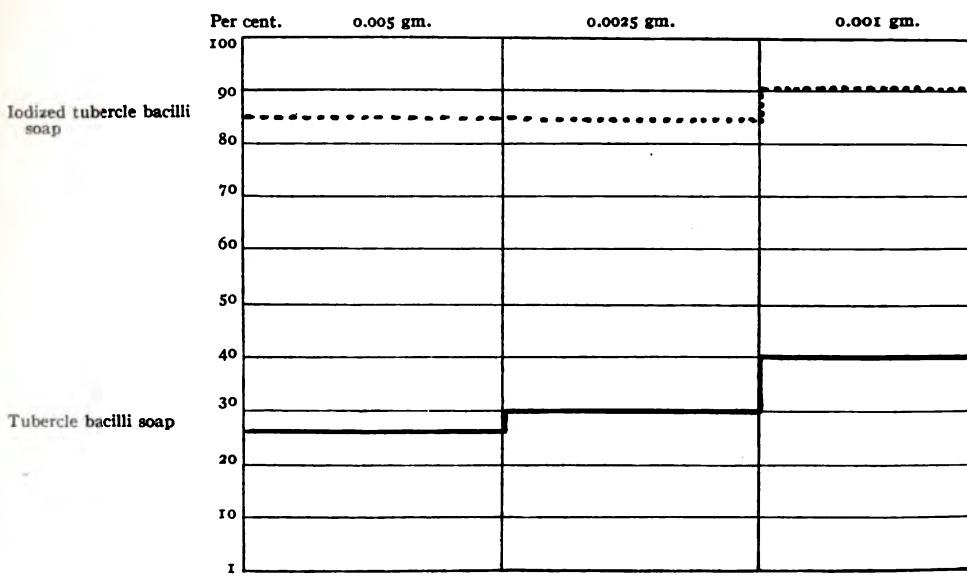
- |                            |                  |                        |
|----------------------------|------------------|------------------------|
| 1 = hempseed soap.         | Iodin value 100. | Solution 1.0 per cent. |
| 2 = linseed soap.          | Iodin value 100. | Solution 1.0 per cent. |
| 3 = croton soap.           | Iodin value 61.  | Solution 1.6 per cent. |
| 4 = olive oil soap.        | Iodin value 44.  | Solution 2.2 per cent. |
| 5 = castor oil soap.       | Iodin value 35.  | Solution 3.0 per cent. |
| 6 = tubercle bacilli soap. | Iodin value 25.  | Solution 4.0 per cent. |
| 7 = cod-liver oil soap.    | Iodin value 92.  | Solution 1.1 per cent. |

TEXT-FIG. 3. Effect of soaps of tubercle bacilli and various other soaps on tryptic digestion.

We reported previously that, with one exception, the activity of the soaps of the unsaturated fatty acids as inhibiting agents was proportionate to their iodin value. We did not include in the experiment described the soap prepared from the extractives of

the tubercle bacilli. This is shown in text-figure 3 in comparison with the other soaps which we have been using. After the iodin value of the respective soaps had been determined, they were made up in such strengths that one cubic centimeter of each had approximately the same iodin value.

A study of text-figure 3 shows that of all the soaps investigated the one prepared from tubercle bacilli is the most active in preventing enzyme action. We had already observed that it was as active as the other soaps in 1 per cent. solutions and were not surprised to find it more so when made up according to its iodin value.



TEXT-FIG. 4. Effect of saturation of unsaturated soaps of tubercle bacilli with iodin.

The method used by us in extracting the acids from the bacilli in preparing the soap does not give any indication of the quantity originally present. On the contrary, it offered every opportunity for the unsaturated acids to become saturated, thus lowering the iodin value and reducing its activity as an inhibiting agent.

In the previous report we showed that the activity of the soaps of the unsaturated fatty acids could be destroyed by saturation with iodin. The soaps so treated did not become entirely inactive.

but this may have been due either to the presence of some free iodin or to incomplete saturation. It is of importance to determine if the soaps prepared from tubercle bacilli are similarly affected when treated with iodin. To five cubic centimeters of a 1 per cent. solution of the soap was added 0.05 of a gram of iodin, and a few crystals of iodide of potassium, and the mixture was permitted to stand over night. The following morning it was shaken repeatedly with chloroform, until fresh portions of the latter remained clear, showing that there was no more free iodin. Text-figure 4 shows the effect of saturating the soap with iodin.

The soap used in this experiment was old and therefore not very active, but the effect of treatment with iodin was evident. The experiment was repeated several times and a similar result obtained in each instance.

Numerous chemicals have been studied in the attempt to find one that will be effective as a therapeutic agent in tuberculosis. Such an agent, in order to be of value, must combine with some constituent of the tubercle bacillus, and our experiments indicate that iodin as a component of the chemical substance may be the means of bringing about such a combination.

We do not know the nature of the unsaturated fatty acids exerting the inhibiting action, but their isolation and identification and further work on their inhibiting action are now under way.

Wells (6) obtained an average of 0.001 of a gram of fatty acids to one gram of caseous matter, which indicates the small amount of soap present in this substance. This finding has little bearing on the relation of the fatty acids of tubercle bacilli to caseation, as the influence of the soaps depends on the amount of ferment present and not on the quantity of protein. That small amounts of the soap are capable of inhibiting enzyme action is shown in text-figure 1, where it was found that in the tube containing 0.001 of a gram of soap the digestion was only half that obtained in the control containing no soap. The influence of soaps as inhibiting agents was well shown in our previous report in which it was demonstrated that the smallest quantity completely inhibiting the standard amount of trypsin materially influences the action of ten times that amount of ferment. In

addition, our experiments indicate that the ferment are destroyed as a result of combining with the soaps.

The soaps are probably formed from the neutral fats of the bacilli. The lipases hydrolyze the fats and the acids are then saponified. In partial support of this view we have the work of Klotz (7) who demonstrated lipases in tuberculous pus, and of Bergel (8) who states that the ferment is present in the lymphoid cells of tubercles while Corper (9) found lipases in tubercle bacilli.

The demonstration that tubercle bacilli contain ferment-inhibiting substances brings us one step nearer a rational explanation of caseation in tuberculosis. The subject will be discussed more fully in a subsequent report.

#### CONCLUSIONS.

1. Tubercl bacilli contain unsaturated fatty acids which, when saponified, have the property of inhibiting the action of trypsin and leucoprotease.
2. In proportion to their iodin value these soaps are more active as inhibiting agents than the soaps prepared from linseed, olive, and cod-liver oils.
3. The activity of the soaps is dependent on the presence of unsaturated carbon bonds.
4. Saturation of the soaps with iodin destroys their inhibiting action.
5. Soaps probably play an important part in the production of the condition known as caseation in tuberculosis.

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## A FURTHER STUDY OF NITROGEN RETENTION IN THE BLOOD IN EXPERIMENTAL ACUTE NEPHRITIS.\*

By HOWARD T. KARSNER, M.D., AND W. DENIS, PH.D.

(From the Laboratory of Pathology (Phillips Fund) and the Laboratory of Biological Chemistry of the Harvard Medical School, Boston.)

In a previous communication<sup>1</sup> we reported the results of the study of experimental acute nephritis in the cat produced by uranium nitrate, by potassium chromate, and by cantharidin. It was shown that these forms of nephritis result in moderate but distinctive retention in the blood of non-protein and urea nitrogen; that from the anatomical point of view the almost purely tubular forms of nephritis result in moderate retention of nitrogen; and that the additional anatomical involvement of the glomerulus leads to a somewhat greater retention. Since the publication of this report the same methods have been applied in other studies. Frothingham, Fitz, Folin, and Denis<sup>2</sup> have shown that the nitrogen retention and phenolsulphonephthalein excretion are parallel and in accordance with the degree of anatomical lesion in uranium nephritis of the rabbit, and that nitrogen retention lags somewhat behind the diminished excretion of the dye. Farr and Austin<sup>3</sup> have shown that in human cases the appearance of chronic passive congestion of the kidneys leads to no increase in the total non-protein nitrogen; that in chronic nephritis associated with marked albuminuria and edema there is little if any increase; that in chronic nephritis with hypertension

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<sup>1</sup> Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat, *Jour. Exper. Med.*, 1912, xvi, 789.

<sup>2</sup> Frothingham, C., Jr., Fitz, R., Folin, O., and Denis, W., The Relation between Non-Protein Nitrogen Retention and Phenolsulphonephthalein Excretion in Experimental Uranium Nephritis, *Arch. Int. Med.*, 1913, xii, 245.

<sup>3</sup> Farr, C. B., and Austin, J. H., The Total Non-Protein Nitrogen of the Blood in Nephritis and Allied Conditions, *Jour. Exper. Med.*, 1913, xviii, 228.

there is a well marked increase in total non-protein nitrogen with percentage increase of the ammonia-urea fraction; and that there is a concordance between the appearance of uremia and increase in the total non-protein nitrogen. Folin, Denis, and Seymour<sup>4</sup> have shown that in cases of chronic interstitial nephritis with high blood pressure it is possible by low protein diet to reduce the total non-protein nitrogen practically to normal, and they state that there "no marked connection between the blood pressure and the degree of retention, indeed it is doubtful whether there is any connection." They also found no close connection between phenolsulphonephthalein excretion and nitrogen retention, presumably because the latter is so much under the influence of diet.

The purpose of the present communication is to present observations of other varieties of experimental acute nephritis than those presented in the first publication, thus including the forms produced by a specific hemolytic immune serum, by sodium arsenate, by diphtheria toxin, and by tartaric acid. The same technique was employed as in the preceding study, and, in addition, as often as possible two determinations of the normal blood were made in each experiment. Unsuccessful attempts were made to produce an experimental nephritis of the cat by the use of rattlesnake venom. A cat weighing 2,500 grams died in thirty-six hours from a hypodermatic injection of 0.010 of a gram of dried venom; a cat weighing 2,200 grams survived an injection of 0.009 of a gram of dried venom; and a cat weighing 3,300 grams survived an injection of 0.013 of a gram of dried venom. None of the animals showed any retention of non-protein nitrogen, and histologically their kidney showed nothing that could be interpreted as nephritis.

<sup>4</sup> Folin, O., Denis, W., and Seymour, M., The Non-Protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis (Arteriosclerosis) as Influenced by the Level of Protein Metabolism, *Arch. Int. Med.*, 1914 (in press); Seymour, M., The Effect of Nitrogenous Waste Products in the Blood in Chronic Interstitial Nephritis, *Boston Med. and Surg. Jour.*, 1913, clxix, 795.

<sup>5</sup> The dried venom of *Crotalus adamanteus* was given us by Dr. Joseph Mac Farland of Philadelphia.

## IMMUNE SERUM NEPHRITIS.

It has been pointed out by Pearce and Eisenbrey<sup>8</sup> that the nephritis produced by a hemolytic immune serum differs from that produced by a specific nephrotoxic serum principally in that the former shows more marked glomerular change histologically. Physiologically, however, there is no notable difference between the two, both showing the vascular reactions of a tubular nephritis. Because of the greater ease of preparation, hemolytic immune serum was used in the present study. In the cat there is, in the early stage of this variety of nephritis, moderate cloudy swelling of the tubular epithelium most noticeable in the distal convoluted tubules associated with moderate swelling of the capillary endothelium of the tuft and the appearance of much albuminous precipitate in the subcapsular space. Hyaline and granular casts are frequent in the ascending loops of Henle, but the picture throughout is obscured by the bile and hemoglobin staining of the tissues. After the disappearance of this staining the glomerular change is somewhat more marked and the degeneration of the distal convoluted tubules persists, in diminishing degree of severity, up to twelve days, the longest period of observation.

*Cat 15.*—Weight 1,650 gm. Given intravenously 0.8 c.c. of specific hemolytic immune serum (about 0.5 c.c. per kilo). Bled on the 2d, 3d, 4th, 8th, 10th, and 12th days. Hemoglobinuria for 24 hours. Albuminuria appeared on the 2d day and continued until the 4th day, but had disappeared on the 8th day. Killed with chloroform on the 12th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (8 dys.)	42 mg. <sup>7</sup>	27 mg.
Before injection (4 dys.)	41 mg.	27 mg.
21 hrs. after injection	52 mg.	35 mg.
45 hrs. after injection	47 mg.	31 mg.
69 hrs. after injection	48 mg.	23 mg.
7 dys. after injection	52 mg.	27 mg.
9 dys. after injection	42 mg.	23 mg.
11 dys. after injection	55 mg.	22 mg.

<sup>8</sup> Pearce, R. M., and Eisenbrey, A. B., A Physiological Study of Experimental Nephritis Due to Bacterial Poisons and Cytotoxic Sera, *Jour. Exper. Med.*, 1911, xiv, 306.

<sup>7</sup> In all cases the figures represent milligrams per 100 c.c. of blood.

*Cat 16.*—Weight 1,950 gm. Given intravenously 1.5 c.c. of specific hemolytic immune serum (about 0.75 c.c. per kilo). Bled on the 2d day. Albuminuria and hemoglobinuria on the 2d day. Killed with chloroform on the 2d day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (3 wks.)	39 mg.	26 mg.
Before injection (2 wks.)	39 mg.	25 mg.
24 hrs. after injection	105 mg.	84 mg.

*Cat 17.*—Weight 2,150 gm. Given intravenously 1.6 c.c. of specific hemolytic immune serum (about 0.75 c.c. per kilo). Bled on the 2d day. Hemoglobinuria and albuminuria on the 2d day. Found dead on the morning of the 3d day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 wk.)	40 mg.	24 mg.
Before injection (4 dys.)	34 mg.	19 mg.
24 hrs. after injection	80 mg.	45 mg.

It will be seen that a relatively small dose of hemolytic immune serum is capable of producing a slight retention of nitrogen in the blood and that larger doses result in well marked retention of nitrogen with a considerable increase in the urea fraction. This is a nephritis which shows extensive tubular change histologically and moderate glomerular change, a nephritis which physiologically is tubular in character, produces a moderate degree of nitrogen retention.

#### ARSENIC NEPHRITIS.

Arsenic produces a nephritis in the cat which in the earlier stages shows histologically only slight glomerular change in the form of albumin in the subcapsular space and an occasional swollen endothelial cell. The tubular epithelium shows a rapidly advancing cloudy swelling, proceeding particularly in the proximal convoluted tubules to marked necrosis; there is much granular and hyaline cast formation. In the later stages (two weeks) the epithelium shows a marked tendency to recovery and the glomerulitis becomes somewhat more distinct but never very severe. This form of nephritis in dogs is primarily vascular in the physiological sense,<sup>8</sup> but in its later stages (three to five days) is more nearly like the tubular forms. Examinations of the urine have shown an increased output

<sup>8</sup> Pearce, R. M., Hill, M. C., and Eisenbrey, A. B., Experimental Acute Nephritis: The Vascular Reactions and the Elimination of Nitrogen, *Jour. Exp. Med.*, 1910, xii, 196.

of nitrogen. The highly fatal character of arsenic intoxication renders the study of the condition somewhat more difficult than in the case of the other nephrotoxic agents.

*Cat 18.*—Weight 2,400 gm. Given subcutaneously 0.025 gm. of sodium arsenate (about 0.010 gm. per kilo). Bled after 6 hours and on the 2d, 5th, and 6th days. No albuminuria up to the 11th day. On the 11th day given 0.075 gm. of sodium arsenate (about 0.030 gm. per kilo). Bled after 18 hours, after showing slight albuminuria; killed with chloroform after bleeding.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 mo.)	40 mg.	20 mg.
Before injection (3 wks.)	40 mg.	21 mg.
3 hrs. after 1st injection	46 mg.	28 mg.
30 hrs. after 1st injection	47 mg.	28 mg.
78 hrs. after 1st injection	41 mg.	23 mg.
102 hrs. after 1st injection	50 mg.	30 mg.
18 hrs. after 2d injection	79 mg.	66 mg.

*Cat 19.*—Weight 2,500 gm. Given subcutaneously 0.075 gm. of sodium arsenate (0.030 gm. per kilo). Bled after 6 hours, at which time moderate albuminuria was found. Found dead at the end of 24 hours.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (3 dys.)	38 mg.	20 mg.
6 hrs. after injection	40 mg.	28 mg.

*Cat 20.*—Weight 2,080 gm. Given subcutaneously 0.045 gm. of sodium arsenate (about 0.0225 gm. per kilo). Bled on the 2d, 3d, 5th, 7th, 8th, and 14th days. Albuminuria appeared on the 2d day and continued until the 7th, but had disappeared on the 14th day. Killed with chloroform on the 18th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (19 dys.)	44 mg.	28 mg.
Before injection (2 wks.)	43 mg.	28 mg.
24 hrs. after injection	63 mg.	50 mg.
48 hrs. after injection	55 mg.	39 mg.
96 hrs. after injection	50 mg.	40 mg.
120 hrs. after injection	47 mg.	30 mg.
2 wks. after injection	39 mg.	17 mg.

*Cat 21.*—Weight 1,850 gm. Given subcutaneously 0.043 gm. of sodium arsenate (about 0.0225 gm. per kilo). Bled on the 2d and 4th days. Albuminuria appeared on the 2d day and continued until death. Animal was found dying on the 5th day, and blood was taken from the posterior vena cava as death occurred.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (6 dys.)	42 mg.	27 mg.
Before injection (5 dys.)	43 mg.	27 mg.
24 hrs. after injection	52 mg.	39 mg.
72 hrs. after injection	125 mg.	98 mg.

*Cat 22.*—Weight 2,000 gm. Given subcutaneously 0.050 gm. of sodium arsenite (0.025 gm. per kilo). Bled on the 2d, 4th, and 10th days. Albuminuria appeared on the 2d day and continued until death. Killed with chloroform on the 10th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	39 mg.	25 mg.
Before injection (3 dys.)	45 mg.	30 mg.
24 hrs. after injection	95 mg.	68 mg.
72 hrs. after injection	40 mg.	27 mg.
216 hrs. after injection	57 mg.	29 mg.

From the results it will be seen that arsenic nephritis is accompanied by a moderate retention of nitrogen in from twenty-four to seventy-two hours, but that in the early stages, when physiologically it is of vascular type the retention is extremely slight. In the animal with most marked nitrogen retention the changes histologically were almost solely tubular, even at a stage when physiologically the condition is a tubular nephritis. From the point of view of nitrogen retention, the nephritis of arsenic shows the most marked retention at the time when the tubular changes are most prominent.

#### DIPHTHERIA TOXIN NEPHRITIS.

The nephritis produced by a single toxic dose of diphtheria toxin is essentially an acute glomerular nephritis.<sup>9</sup> There is swelling of the glomerular tuft with slight endothelial proliferation, infiltration of the polymorphonuclear leucocytes, occasionally pyknotic nuclei, albumin in the subcapsular space, occasionally hyaline thrombi, and swelling of the subcapsular epithelium. The tubular epithelium shows moderate cloudy swelling in the earlier cases with the accumulation of a moderate amount of granular material in the lumina of the tubules. Later there is more marked degeneration and small areas of actual necrosis. The change affects all the tubular epithelium, but most markedly that of the proximal convoluted tubules.

For the sake of uniformity the dose is given in units. It has been shown by Paton, Dunlop, and Macadam<sup>10</sup> that there is an in-

<sup>9</sup> The toxin used was furnished and titrated by Dr. Theobald Smith.

<sup>10</sup> Paton, N., Dunlop, J. C., and Macadam, I., On the Modifications of the Metabolism Produced by the Administration of Diphtheria Toxin, *Jour. Physiol.*, 1899, xxiv, 331.

creased output of nitrogen following the administration of diphtheria toxin, which they believe to be due to the increased catabolism of fever, but there is nothing in their report to indicate whether or not they gave sufficiently large doses to produce a distinct nephritis. Pearce and Eisenbrey<sup>11</sup> regard the earlier stages of the nephritis as physiologically tubular in character and the later stages as probably vascular.

*Cat 23.*—Weight 2,850 gm. Given 10 units of diphtheria toxin subcutaneously (about 3 units per kilo). Bled on the 2d day. Well marked albuminuria on the 2d day. Found dead on the morning of the 4th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	41 mg.	25 mg.
Before injection (1 dy.)	42 mg.	28 mg.
24 hrs. after injection	50 mg.	33 mg.

*Cat 24.*—Weight 2,350 gm. Given 4.5 units of diphtheria toxin subcutaneously (about 2 units per kilo). Bled on the 3d and 5th days. No urine at the end of 24 hours; did not eat well after injection. Slight albuminuria at the end of 48 hours, increasing in amount until death. Found dead on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	38 mg.	22 mg.
Before injection (same dy.)	38 mg.	23 mg.
48 hrs. after injection	61 mg.	39 mg.
96 hrs. after injection	227 mg.	186 mg.

*Cat 25.*—Weight 2,950 gm. Given 9 units of diphtheria toxin subcutaneously (about 3 units per kilo). Bled on the 3d and 4th days. Albuminuria appeared on the 3d day (urine lost on the 2d day), and was well marked until death. Found dead on the morning of the 5th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 wk.)	41 mg.	24 mg.
Before injection (3 dys.)	41 mg.	26 mg.
48 hrs. after injection	50 mg.	36 mg.
72 hrs. after injection	200 mg.	158 mg.

*Cat 26.*—Weight 2,700 gm. Given 3 units of diphtheria toxin subcutaneously (about 1 unit per kilo). Bled on the 3d and 5th days. Albuminuria appeared on the 3d day and was moderate until death. Found dead on the afternoon of the 5th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	34 mg.	17 mg.
48 hrs. after injection	80 mg.	45 mg.
96 hrs. after injection	254 mg.	216 mg.

<sup>11</sup> Pearce, R. M., and Eisenbrey, A. B., *loc. cit.*

It will be seen that in the earlier stages, when the condition physiologically is tubular, there is only slight retention of nitrogen, but that later, when physiologically the condition is vascular the retention becomes extremely marked. This latter effect, however, probably has much to do with the increased catabolism of the intoxication, and, as Pearce and Eisenbrey point out, the vascular reactions are influenced at this stage by the profound and general effect of the toxin rendering accurate physiological conclusions impossible. From both histological and physiological studies there can be little doubt that there is severe glomerular change at this period of great retention, as well as distinct tubular change.

#### TARTARIC ACID NEPHRITIS.

That tartaric acid can produce a nephritis was first pointed out by Underhill,<sup>12</sup> confirmed and studied further by Underhill, Wells and Goldschmidt,<sup>13</sup> and also confirmed by Pearce and Ringer.<sup>14</sup>

The nephritis produced in the cat by the subcutaneous injection of tartaric acid (racemic, Merck) shows a most marked necrosis of the epithelium of the convoluted tubules, as described in dogs and rabbits by Underhill and Wells. There is cloudy swelling of the epithelium generally. There is much albuminous precipitate in the subcapsular space of the glomerulus, as described by Pearce and Ringer. In the later stages the epithelial change is only that of slight cloudy swelling of the epithelium of the proximal convoluted tubule and occasional glomerular tufts show slight swelling of the convoluted tubules. Two cases show well marked interstitial edema. Studies of the vascular reactions of this form of nephritis have not appeared.

<sup>12</sup> Underhill, F. P., The Influence of Sodium Tartrate upon the Elimination of Certain Urinary Constituents during Phlorhizin Diabetes, *Jour. Biol. Chem.* 1912, xii, 115.

<sup>13</sup> Underhill, F. P., Wells, H. G., and Goldschmidt, S., Tartrate Nephritis with Especial Reference to Some of the Conditions under Which It May Be Produced, *Jour. Exper. Med.*, 1913, xviii, 322; A Study of Renal Secretion during Tartrate Nephritis, *ibid.*, p. 347; A Note on the Fate of Tartrates in the Body, *ibid.*, p. 317.

<sup>14</sup> Pearce, R. M., and Ringer, A. I., A Study of Experimental Nephritis Caused by the Salts of Tartaric Acid, *Jour. Med. Research*, 1913, xxix, 57.

*Cat 27.*—Weight 1,650 gm. Given subcutaneously 0.75 gm. of tartaric acid in 6 c.c. of water, plus 4 c.c. of saturated sodium carbonate solution (about 0.45 gm. of tartaric acid per kilo). Bled on the 2d, 3d, 4th, 6th, 7th, 8th, 11th, and 13th days. Albuminuria appeared on the 2d day, but was absent on the 5th, 6th, and 7th days, reappeared on the 8th, 9th, and 10th days, and was absent on the 13th day. Killed with chloroform on the 14th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	27 mg.
Before injection (same dy.)	42 mg.	24 mg.
22 hrs. after injection	69 mg.	45 mg.
46 hrs. after injection	36 mg.	20 mg.
71 hrs. after injection	40 mg.	22 mg.
118 hrs. after injection	57 mg.	31 mg.
142 hrs. after injection	54 mg.	30 mg.
11 dys. after injection	50 mg.	30 mg.
13 dys. after injection	52 mg.	31 mg.

*Cat 28.*—Weight 2,150 gm. Given subcutaneously 1 gm. of tartaric acid in 5 c.c. of water, plus 7 c.c. of saturated sodium carbonate solution (about 0.5 gm. of tartaric acid per kilo). Bled on the 2d and 3d days. Albuminuria appeared on the 2d day and continued on the 3d day. Found dead in cage on the morning of the 4th day probably as the result of heart puncture.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	26 mg.
Before injection (same dy.)	46 mg.	27 mg.
24 hrs. after injection	154 mg.	116 mg.
45 hrs. after injection	222 mg.	181 mg.

*Cat 29.*—Weight 2,120 gm. Given subcutaneously 1.8 gm. of tartaric acid in 8.2 c.c. of water, plus 10 c.c. of saturated sodium carbonate solution (about 0.9 gm. of tartaric acid per kilo). Bled on the 2d, 3d, 5th, 6th, 7th, 8th, 11th, and 13th days. Albuminuria appeared on the 2d day, continued very small in amount, was barely perceptible on the 8th day, and became marked thereafter. Cat developed snuffles and was killed on the 13th day.

Time of bleeding	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	26 mg.
Before injection (same dy.)	44 mg.	26 mg.
24 hrs. after injection	132 mg.	100 mg.
45 hrs. after injection	94 mg.	66 mg.
96 hrs. after injection	38 mg.	19 mg.
120 hrs. after injection	37 mg.	19 mg.
144 hrs. after injection	40 mg.	25 mg.
7 dys. after injection	40 mg.	23 mg.
10 dys. after injection	50 mg.	30 mg.
12 dys. after injection	50 mg.	29 mg.

*Cat 30.*—Weight 1,800 gm. Given subcutaneously 1.6 gm. of tartaric acid in 8 c.c. of water, plus 8 c.c. of saturated sodium carbonate solution (0.9 gm. of

tartaric acid per kilo). Bled on the 2d, 3d, 4th, and 6th days. Albuminuria appeared on the 2d day and continued until death. Found dead in cage on the morning of the 7th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	40 mg.	22 mg.
Before injection (same dy.)	41 mg.	24 mg.
24 hrs. after injection	166 mg.	120 mg.
48 hrs. after injection	229 mg.	190 mg.
73 hrs. after injection	156 mg.	119 mg.
129 hrs. after injection	130 mg.	100 mg.

*Cat 31.*—Weight 2,200 gm. Given subcutaneously 2 gm. of tartaric acid 10 c.c. of water, plus 10 c.c. of saturated sodium carbonate solution (about 1 gm. of tartaric acid per kilo). Bled on the 2d and 3d days. Albuminuria appeared on the 2d day and on the morning of the 4th day. Complete anuria at the end of 24 hours, and on the 2d and 3d days. Found dead in cage on the morning of the 4th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	40 mg.	26 mg.
22 hrs. after injection	133 mg.	100 mg.
48 hrs. after injection	210 mg.	160 mg.

*Cat 31.*—Weight 2,200 gm. Given subcutaneously 2 gm. of tartaric acid 9 c.c. of water, plus 6 c.c. of saturated sodium carbonate solution (about 1 gm. of tartaric acid per kilo). Bled on the 2d, 4th, and 6th days. Albuminuria appeared on the 2d day and was marked until death. Developed snuffles and was killed with chloroform on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	47 mg.	30 mg.
24 hrs. after injection	50 mg.	32 mg.
72 hrs. after injection	277 mg.	218 mg.
120 hrs. after injection	382 mg.	312 mg.

It can be seen that the degree of nitrogen retention in these animals with tartaric acid nephritis is well marked, that it appears to bear a direct ratio to the dose of tartaric acid, and that it is more marked when the lesion histologically is almost purely tubular.

#### SUMMARY.

These studies, like all studies of the kidney, are difficult of interpretation because of the impossibility of producing a pure glomerular or pure tubular nephritis. Arsenic nephritis in its early stage is physiologically a vascular nephritis, but anatomically tubular, and shows in this stage almost no retention of nitrogen, although studies of urinary nitrogen indicate an increased catabolism. On the other hand, diphtheria nephritis in the early stages is anatomically

nephritis with marked involvement of the glomerulus anatomically, but it is physiologically a tubular form, and in this early stage there is little or no nitrogen retention although studies of metabolism indicate that protein catabolism is increased by the administration of diphtheria toxin. In both cases, however, there appears to come a time when the excreting power of the kidney is exhausted and nitrogen accumulation occurs in the blood. This is much less marked in the arsenic nephritis, with less tubular change, than in diphtheria toxin nephritis with its marked tubular degeneration. This same late accumulation of blood nitrogen is seen in immune serum nephritis, where the tubular changes are persistent and relatively more severe than the glomerular change. It might well be said that this is no argument for the influence of the tubules in the excretion of waste nitrogen because of the general exhaustion of the organism as a whole and of the kidney in particular, and that no positive conclusions can be drawn is indicated by the opening sentence of this summary. Tartrate nephritis was at first considered as purely tubular; but the appearance of occasional glomerular change, as mentioned by Wells in one of his animals, and the fact that the presence of precipitated albumen in the subcapsular space, pointed out by Pearce and Ringer, probably indicates an increased permeability of the tuft capillaries, all lead to the conclusion that although the tubular change predominates, there is, possibly, slight alteration of the glomerulus. This form of nephritis shows the most marked retention of nitrogen, persisting even though the glomeruli show almost no change, tending to clear up with the progress of time and evidently also with the repair of the tubular change, and bearing a direct relation to the dose of tartaric acid and presumably with the degree of tubular change. It must be remembered, however, that the nephritis in the cases of greatest retention is a very severe form, and this again clouds the physiological interpretation of the results.

The study shows no reason for altering the conclusions of our earlier studies,<sup>15</sup> but from the interpretation accorded above it appears to throw more stress on tubular change as determining nitrogen retention. It confirms in addition the value of the methods used for studies of this type.

<sup>15</sup> Folin, O., Karsner, H. T., and Denis, W., *loc. cit.*

## A NOTE ON NITROGEN RETENTION FOLLOWING REPEATED INJECTIONS OF NEPHROTOXIC AGENTS.\*

By HOWARD T. KARSNER, M.D., AND W. DENIS, PH.D.

(*From the Laboratory of Pathology (Phillips Fund) and the Laboratory of Biological Chemistry of the Harvard Medical School, Boston.*)

During the course of experiments previously reported<sup>1</sup> it was considered advisable to try to produce a subacute or chronic nephritis by the repeated injection of nephrotoxic agents and to observe the effects of the repeated injections on the amount of non-protein nitrogen in the blood. For this purpose uranium nitrate and diphtheria toxin were selected, and the technique detailed in the first study was followed. The experiments although not extensive are considered of sufficient interest to justify publication. An animal in which spontaneous chronic nephritis had occurred is included in the report because of the relation of this process to the experimental conditions studied and because of the close relation it bears to the work of Folin, Denis, and Seymour,<sup>2</sup> which shows that in human cases of chronic interstitial nephritis with hypertension it is possible to reduce the blood nitrogen practically to normal by the use of a low protein diet.

### URANIUM NITRATE.

*Cat 33.*—Weight 2,670 gm. Given 0.00025 gm. of uranium nitrate daily (about 0.0001 gm. per kilo) for 5 consecutive days; then given double this dose for 5 consecutive days; then triple the primary dose for 5 consecutive days; and then

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<sup>1</sup> Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat. *Jour. Exper. Med.*, 1912, xvi, 78.  
Karsner, H. T., and Denis, W., A Further Study of Nitrogen Retention in the Blood in Experimental Acute Nephritis, *idem*, 1914, xix, 259.

<sup>2</sup> Folin, O., Denis, W., and Seymour, M., The Non-Protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis (Arteriosclerosis) as Influenced by the Level of Protein Metabolism, *Arch. Int. Med.*, 1914 (in press). Seymour, M., The Effect of Nitrogenous Waste Products in the Blood in Chronic Interstitial Nephritis, *Boston Med. and Surg. Jour.*, 1913, clxix, 795.

quadruple the primary dose for 7 consecutive days. The cat lived for 11 days more and was found dead in the cage on the morning of the 41st day. It was observed for 41 days and received a total of 0.018 gm. of uranium nitrate, 9 times the dose necessary to produce a marked nephritis in a 4,000 gm. cat. Albuminuria appeared to a slight degree on the 3d day, becoming marked 24 hours after doubling the dose, and continuing so until the dose was discontinued, when it became slight and remained so until death.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	43 mg.	28 mg.
3d dy. of experiment	43 mg.	27 mg.
5th dy.	40 mg.	25 mg.
Dose doubled		
7th dy.	40 mg.	25 mg.
9th dy.	40 mg.	26 mg.
11th dy.	40 mg.	25 mg.
14th dy.	41 mg.	25 mg.
16th dy.	41 mg.	22 mg.
18th dy.	43 mg.	28 mg.
Triple dose (18th dy.)		
23d dy.	53 mg.	36 mg.
Quadruple dose (23d dy.)		
25th dy.	54 mg.	39 mg.
28th dy.	91 mg.	67 mg.
30th dy.	126 mg.	113 mg.
Dose discontinued		
32d dy.	160 mg.	125 mg.
35th dy.	174 mg.	142 mg.
39th dy.	228 mg.	190 mg.

Found dead on the morning of the 41st day.

At autopsy there was present a slight acute valvulitis (mitral), probably the mechanical effect of the frequent heart punctures, a chronic adhesive pericarditis from the same cause, fat necrosis in the abdominal fat, but an apparently normal pancreas. The kidneys showed no change except that the glomeruli were white and prominent. Histologically the interstitial tissue shows between the tubules of the cortex numerous small areas of recent overgrowth, the nuclei being large, prominent, and vesicular. The glomeruli show a normal capsule and normal capsular epithelium. The subcapsular space is filled with a hyaline albuminous precipitate. The vascular tuft occupies about the normal space in the glomerulus but is almost devoid of blood; the endothelium shows extremely large frequent and vesicular nuclei, occasional mitotic figures, occasional lymphocytes and leucocytes and a well marked increase of collagenous intercellular material, apparently representing thickened capillary walls. Occasional proximal convoluted tubules are normal, but many more show hyaline casts and granular detritus within the lumen; in these tubules the epithelium does not project the normal distance above the basement, is narrowed, but shows no necrosis. The other tubules, particularly the loops of Henle and the distal convoluted tubules, show

an unusually large number of nuclei, in some cases there being two and even three nuclei in what appears to be a single cell body, although very few mitotic figures could be found. The blood vessels appear to be normal. The condition is interpreted as a subacute diffuse nephritis.

This animal had in the beginning functionally normal kidneys which were also probably anatomically normal, for at the end of the experiment they showed neither grossly nor histologically the features of a spontaneous chronic nephritis. The repeated doses of uranium nitrate resulted in the appearance on the third day of albuminuria, which in this case can safely be interpreted as indicating functionally at least the presence of a nephritis. Not until after fifteen days had elapsed, however, was any demonstrable nitrogen retention observed and then after four daily administrations of triple the primary dose or 0.0003 of a gram of uranium nitrate per kilo, a single dose of 0.0005 of a gram being sufficient to produce well marked nephritis in the cat.<sup>8</sup> This moderate retention continued until five days after the primary daily dose was quadrupled. For nine days after the drug was discontinued the nitrogen retention increased, a period during which the nephritis functionally became more marked, but in which the anatomical condition of the kidney, at least in so far as the epithelium was concerned, showed attempts at repair. It is hardly probable that the drug was retained in the body during the twelve days following the cessation of administration and hence the nitrogen retention and the final death of the animal must be looked upon as the effects of the artificially produced nephritis, even though the course of the experiment appeared to show nitrogen retention in a general way proportional to the dose of drug.

#### DIPHTHERIA TOXIN.

Three animals were given diphtheria toxin<sup>4</sup> in daily doses of 0.25, 0.5, and 0.5 of a unit per kilo respectively.

*Cat 34.*—Weight 3,050 gm. Given subcutaneously 0.3 of a unit of diphtheria toxin (about 0.1 of a unit per kilo) daily for 30 days with the exception of the 6th, 13th, and 26th days, making a total of 27 injections, or 8.1 units of toxin. Albuminuria appeared on the 7th day, after the administration of a total of 1.5 units of toxin, became marked on the 13th day, after the animal had received

<sup>8</sup> Folin, Karsner, and Denis, *loc. cit.*, p. 790 (cat 3).

<sup>4</sup> The toxin was furnished us and titrated by Dr. Theobald Smith.

total of 3.3 units of toxin, and continued well marked until it was killed by chloroform.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	35 mg.	16 mg.
4th dy. of experiment	37 mg.	20 mg.
7th dy.	37 mg.	20 mg.
9th dy.	35 mg.	19 mg.
11th dy.	36 mg.	19 mg.
14th dy.	50 mg.	28 mg.
18th dy.	40 mg.	24 mg.
25th dy.	46 mg.	28 mg.
31st dy.	41 mg.	28 mg.

The autopsy showed nothing notable grossly other than the kidneys which were small, firm, and showed slight depressions in the outer surface along the lines of the larger veins; the cut surface showed almost no striations and the glomeruli were prominent and red. Histologically there is found no overgrowth of connective tissue about the surface veins or in any other part of the kidney. The glomeruli show well marked swelling of the capsular epithelium, and in many cases slight proliferation of the cells, but in no case are there found the crescentic figures of chronic capsular disease. The vascular tufts are filled with blood and although an occasional migrating leucocyte is found the tufts are practically normal. The subcapsular space contains a granular albuminous precipitate. The proximal convoluted tubules are normal except for occasional small areas of necrosis of the epithelium. The distal convoluted tubules show much formation of hyaline droplets in the epithelial cells. The tubules otherwise show finely granular albuminous material in the lumina and the larger collecting tubules show large numbers of hyaline casts. The blood vessels show nothing other than congestion. The condition is a subacute epithelial nephritis with a subacute capsular glomerulitis.

*Cat 35.*—Weight 2,850 gm. Given subcutaneously 0.7 of a unit of diphtheria toxin (about 0.25 of a unit per kilo) daily for 7 days. Bled on the 3d, 5th, and 8th days. Died on the 8th day as the result of heart puncture. Albuminuria appeared on the 4th day and continued in considerable quantity until death.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 mo.)	41 mg.	24 mg.
Before injection (3 wks.)	41 mg.	23 mg.
In the interval the cat lost weight and was ill, but recovered about 1 wk. before continuation of the experiment.		
Before injection (same dy.)	37 mg.	23 mg.
48 hrs. after beginning of injection	37 mg.	23 mg.
96 hrs. after beginning of injection	37 mg.	18 mg.
168 hrs. after beginning of injection	180 mg.	144 mg.

The autopsy performed immediately after death showed nothing notable grossly. The kidneys show histologically a well marked acute change. Through-

out the organ there is a distinct perivascular infiltration of leucocytes, lymphocytes, and endothelial cells. The glomeruli show swelling, desquamation of capsular epithelium, and occasionally pyknosis and karyorrhexis. The subcapsular space contains granular albuminous material and desquamated cells. Capillary tufts although containing blood show endothelial swelling, an occasional mitotic figure, karyorrhexis, and many leucocytes both within the glomerular laries and infiltrating into the tuft. The tubules show slight albuminous degeneration, particularly in the distal convoluted tubules, and the collecting tubules show a few hyaline casts. The blood vessels are normal, except that some of the smaller ones contain an excess of leucocytes. The condition is an acute diffuse nephritis with the most marked change in the glomerulus.

*Cat 36.*—Weight 2,270 gm. Given subcutaneously 2.5 units of diphtheria toxin (about 0.5 of a unit per kilo) daily for 5 days. Bled on the 3d and 5th day. Albuminuria on the 3d day, continuing until death. Found dead in cat on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	50 mg.	38 mg.
48 hrs after first injection	40 mg.	26 mg.
93 hrs. after first injection	222 mg.	182 mg.

The autopsy showed nothing notable grossly. Histologically the kidney differed from that of cat 35 only in that there is much less infiltration of leucocytes in the glomerular tuft and in the perivascular tissue of the organ.

In cats 35 and 36 the repetition of doses produced a greater degree of retention than the single dose would have produced, but no notable retention did not appear before the third day, as was the case with the cats reported previously, which had been given a single larger dose of toxin. These two cats had a severe acute nephritis. Cat 34 with smaller doses developed albuminuria about a week before there was any nitrogen retention; the retention was then only slight and returned to normal in four days, even though there was a continued albuminuria, and the autopsy showed a chronic nephritis of considerable duration. Anatomically this nephritis with its slight retention differed little in extent of involvement from that seen in the other two cats, although the functional powers were widely different. The length of time occupied in the experiments with cat 34 was more than sufficient for the development of immunity; hence the metabolic effects of the later doses would probably have little effect on protein catabolism and thus give less reason for calling on the kidney to excrete large amounts of nitrogenous waste. It would seem probable, therefore, that in acute diphtheria

nephritis the large final accumulation of nitrogen is dependent not only on the presence of the nephritis but to a large degree upon the increased protein catabolism.<sup>5</sup>

#### SPONTANEOUS NEPHRITIS.

*Cat 37.*—Weight 3,500 gm. On first examination and throughout the experiment the animal showed distinct albuminuria, but there were no casts in the urine. A diet of 80 gm. of chopped beef maintained a fairly constant weight. Since albuminuria was constant it will not be mentioned in the following notes.

Date.	Weight.	Diet.	Blood.	
			Non-protein nitrogen.	Urea nitrogen.
Apr. 15	3,500 gm.	80 gm. meat	—	—
Apr. 17		80 gm. meat	69	35
Apr. 25		80 gm. meat	58	32
Apr. 26	3,400 gm.	160 gm. meat	—	—
Apr. 27		160 gm. meat	63	38
Apr. 29		160 gm. meat	66	50
May 1	3,600 gm.	160 gm. meat	67	50
May 3		50 gm. rice, 25 c.c. 15 per cent. cream	—	—
May 7		Same diet	54	40
May 14		(Diarrhea)	—	—
May 15		80 gm. meat; condition cleared up	—	—
May 21	3,200 gm.	80 gm. meat	72	56
May 23		80 gm. meat, 1 gm. sodium chloride	—	—
May 31		80 gm. meat, 1 gm. sodium chloride	54	40
June 1		80 gm. meat	—	—
June 22	Experiment terminated. Animal killed with chloroform.			

At autopsy the heart was enlarged and had a distinctly thickened endocardium. The aorta was dilated in the ascending part. The liver showed a fatty deposit in the centers of the lobules. The kidneys were normal on the outer surface, but on the cut surface showed radial streaks of connective tissue in the cortex, especially prominent near the pyramid.

Histologically the gross findings in the heart and liver are confirmed. The kidney shows a slight overgrowth of connective tissue especially about the glomerular capsules and the blood vessels; associated in many places is moderate infiltration of lymphocytes. The glomerular capsules are slightly thickened and the capsular epithelium is normal. The subcapsular spaces contain a granular albuminous precipitate. The tufts are enlarged and show a distinct increase in

<sup>5</sup> Paton, N., Dunlop, J. C., and Macadam, I., On the Modifications of the Metabolism Produced by the Administration of Diphtheria Toxin, *Jour. Physiol.*, 1899, xxiv, 331.

the number of nuclei; there is almost no blood in the tufts and the collagenous material is increased in amount, and often hyaline. The tubules are normal except that there is a moderate amount of albuminous precipitate in the lumen. The distal convoluted tubules show thinning and increased granulation of the epithelium, and occasional hyaline casts are found in the collecting tubules. This condition is a slight chronic diffuse nephritis with slightly greater quantitative involvement of the glomerular tuft than of other parts of the organ.

This animal as it first came to the laboratory presented in notable degree the features of a spontaneous nephritis. Under the favorable conditions of the laboratory the degree of nitrogen retention sank considerably during the course of one week, and then with several days of excessive protein diet rose slightly, especially in the urea fraction. Four days of extremely low protein diet resulted in a decrease of the total non-protein nitrogen, but still with a relatively high urea fraction. The diet had to be discontinued because a diarrhea developed. Quite unexpectedly, a week of normal diet resulted in an excessive accumulation of nitrogen, and then the addition of sodium chloride in small quantities was followed by a decrease in retained nitrogen. This study is suggestive in connection with the studies of Folin, Denis, and Seymour,<sup>6</sup> but it is not conclusive. The important facts demonstrated are that a mild chronic nephritis in the cat may be accompanied by a moderate retention of nitrogen, that the degree of retention is variable, and that moderate low and extremely low protein diets may be followed by a reduction in the non-protein nitrogen of the blood.

#### SUMMARY.

It is possible by means of repeated injections of uranium nitrate to produce in the cat a subacute or chronic nephritis which can progress to a fatal termination and show in its course increasing accumulation of non-protein nitrogen in the blood. This nephritis differs only slightly from the spontaneous chronic nephritis of the species. Repeated doses of diphtheria toxin produce a subacute form of nephritis with only temporary or slight retention, but nevertheless anatomically a well defined nephritis. The spontaneous nephritis studied resembles more closely that produced by uranium nitrate than that produced by diphtheria toxin both histological and from the standpoint of blood analysis.

<sup>6</sup> Folin, Denis, and Seymour, *loc. cit.*

## A NOTE ON THE SPECIFICITY OF CYTOTOXINS.\*

By ROBERT A. LAMBERT, M.D.

(*From the Department of Pathology of the College of Physicians and Surgeons,  
Columbia University, New York.*)

Since Metchnikoff and Landsteiner, working independently, demonstrated in the serum of rabbits treated with guinea pig spermatozoa the development of substances which immobilized and dissolved spermatozoa more or less completely, many attempts have been made to obtain cytotoxic sera specific for the different types of body cells.

It was early shown by von Dungern (1) that if rabbits were injected with the tracheal epithelium of cattle, the serum of the treated animals developed the power of causing the rapid disintegration of epithelial cells of that kind. He showed further that the serum was lytic for bovine erythrocytes, but that the antibodies possessed a greater affinity for tracheal epithelium. Metchnikoff (2) found that his spermatotoxic serum was likewise hemolytic, but he maintained that the hemolytic power developed as the result of the injection of hemolytic receptors. Later investigators have obtained cytotoxic sera by injecting into animals of a foreign species the cells of practically every tissue of the body; leucocytes (Metchnikoff (3), Besredka (4)), liver (Delezenne (5), Deutsch (6)), kidney (Lindemann (7), Néfédieff (8), Bierry (9), Ascoli and Figari (10)), pancreas (Surmont (11)), adrenal (Bigart and Bernard (12)), thyroid (Gontscharukov (13), Mankovski (14)), heart muscle (Centanni (15)), ovary (Ceconi and Robecchi (16)), nervous tissue (Delezenne (17)), (Centanni (18)), syncytium (Ascoli (19), Liepmann (20)), etc.

Several methods have been employed for testing the immune sera as regards their specificity for the particular cells used as antigens. The methods of exposing cell emulsions to the action of the serum, as used by von Dungern in his trichotoxin studies, and by Flexner and Noguchi (21) in studies upon the action of snake venom on various organ cells, has not been found satisfactory for testing the specificity of cytotoxins. This may be due to the fact that one is dealing with the action of antibodies on dead and dying cells instead of on living functioning ones.

The technique which has been generally used consists in injecting the immune serum into the animal body, either subcutaneously, intraperitoneally, intravenously, or into arteries leading to particular organs. Functional disturbances and histological changes in various organs have served as criteria for determining the degree of specificity. More recently several other methods have been used, especially complement deviation and the epiphanin reaction. The results with the use of these methods are briefly summarized below.

Cytotoxic sera obtained by injecting the cells of an organ into an animal of a

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foreign species are relatively specific for the species from which the antigen was derived; that is, serious or fatal symptoms develop when animals of that species are treated with suitable quantities of the serum. Animals of other species are not, as a rule, so affected. These antibodies are spoken of as showing species specificity. Reports are conflicting as to whether or not they are to any degree specific for the particular organs or tissues used as antigens. That an absolute organ specificity does not exist is generally admitted, but many authors maintain, especially from a study of the lesions found in injected animals, that a relative specificity can be demonstrated. Lüdke and Schüller (22) for example, in a recent paper describe the production in dogs of a true nephritis with insignificant lesions in organs other than the kidney by the injection of the serum of rabbits immunized with dog kidney. Pearce (23), however, called attention to the fact, several years ago, that often the most striking lesion that one finds after the injection of a cytotoxic serum may be referable not to a direct toxic action on certain parenchymatous cells, but to the hemagglutinative and hemolytic properties of the serum, causing thrombosis, embolism, and hemorrhages, with secondary necroses in organs. Pearce argues further that since the cells of different organs evidently have certain receptor characteristics in common, it is hardly conceivable that specific somatogenic cytotoxins can be produced.

Beebe (24) has suggested that by the use of nucleoproteids instead of whole cells as antigens a closer approach to specificity can be reached. Wells (25), however, has recently pointed out that Beebe's suggestion is based on the false assumption that the nucleoproteids constitute the most important and most specific part of the cell.

Apart from its biological interest the problem is important on account of its bearing on the possibility of treating certain diseases, particularly cancer and exophthalmic goitre, with specific cytotoxic sera.

#### EXPERIMENTAL PART.

We have used a technique which appears to possess some advantages over previous methods; namely, the cultivation of tissue *in vitro*. By this method, as was suggested in a former communication (26), active living cells may be exposed to the action of immune sera, under conditions where the effects of such disturbing factors as agglutination, thrombosis, and hemorrhages can be eliminated.

Unfortunately the tissues that have been generally used by workers with other methods—liver, kidney, and other parenchymatous organs,—can not be cultivated satisfactorily in tissue cultures and therefore can not be utilized in experiments with the new technique. After a consideration of the tissues that might be used, rat sarcoma and rat embryo skin were chosen.

The next consideration was the choice of a foreign species which could be used for immunization against these tissues. It was necessary that two conditions be fulfilled: first, the plasma from normal animals of the species should be a suitable culture medium for rat tissues; and secondly, the animals should react well to the injections, that is, they should develop a strong cytotoxic serum. Guinea pigs fulfilled these two conditions. Two groups of guinea pigs were therefore injected, one with sarcoma, the other with rat embryo skin. Plasma from the two sets of animals was later used for tissue cultures for both sarcoma and skin.

*Experiment 1.*—The tissue for injection was prepared as follows: A sarcoma rat was bled to death and the tumor removed aseptically. The tumor tissue, which was extremely friable but not necrotic was cut up with scissors and washed with physiological salt solution to remove the excess of blood. The embryo skin was obtained from embryos near term. The skin was carefully dissected off, washed in salt solution, and finely divided. Very little blood was present.

Guinea pig 1 was inoculated subcutaneously through a Bashford needle with 0.15 gm. of rat sarcoma. Guinea pig 2 received a similar amount of embryo skin. Ten days later a second injection in each animal was made. Twelve days after the second injection each animal was bled according to the technique previously described (27) and plasma was obtained for tissue cultures. A normal guinea pig was also bled to obtain plasma for control preparations. Six series of preparations were made composed of rat sarcoma and rat embryo skin in plasma from each of the three guinea pigs. Each series was composed of fifteen preparations. They were incubated at 37° C. and examined daily. The controls of both sarcoma and skin in normal guinea pig plasma showed after twenty-four hours an active outwandering of cells among which mitotic figures were frequent. At the end of forty-eight hours growth was luxuriant in practically every preparation. The preparations containing immune plasma were, on the contrary, much less active, though the majority showed, especially on the third and fourth days, a fair outwandering of cells. A slight but definite difference was noted in the behavior of both kinds of tissue in the plasma from the two treated animals. For each tissue there was a more marked inhibition of growth in the plasma from guinea pig 1, injected with sarcoma, than in the plasma from the animal treated with embryo skin.

*Experiment 2.*—In order to secure a higher degree of immunization than was obtained in the first experiment, it was decided to use larger doses of tissue for injection. Guinea pig 3 received subcutaneously 0.65 gm. of rat sarcoma. Guinea pig 4 received a similar quantity of embryo skin, prepared as in experiment 1. Twelve days later plasma was obtained in the usual way from each animal, and from a third control normal guinea pig. Small pieces of sarcoma and skin were put up in the three kinds of plasma, making six sets, each composed of fifteen preparations. Examinations were made at the end of twelve hours and daily up to the sixth day of incubation.

The controls of both tissues grew well as usual, active outwandering of cells being noticed at the end of twelve hours. There was marked inhibition in the preparations of all four sets containing immune plasma. Many of the pieces of sarcoma and skin in plasma from both immune animals not only showed no growth but presented an amorphous granular appearance. Some of the preparations, however, showed after two days an outwandering of a small number of cells. As in experiment 1, a definite difference in the toxic power of the two immune plasmas was observed. In this instance, however, the more toxic plasma was that obtained from guinea pig 4, immunized with embryo skin. This plasma acted more strongly on both sarcoma and skin than the plasma from guinea pig 3, as shown by the more complete disintegration of the tissue fragments and the smaller number of cells which wandered out in the preparations that were positive.

*Experiment 3.*—The two guinea pigs used in experiment 2 received after bleeding second injections of 0.65 gm. of sarcoma and skin respectively. Ten days later they were again bled and their plasma was used for cultures of sarcoma and skin as before, with controls in normal guinea pig plasma. All the fragments of both sarcoma and skin in the immune plasma of both series showed rapid disintegration with no outwandering of cells. The controls showed excellent growths.

*Experiment 4.*—A guinea pig was injected intraperitoneally with 15 c.c. of defibrinated rat blood. Twenty-one days later plasma was obtained and used for culture preparations of sarcoma and skin. All preparations in normal guinea pig plasma showed active growth. Both tissues showed a feeble growth in a majority of the preparations in immune plasma. In the remaining negative preparations the disintegration of the tissue fragments was not so marked as in experiments 2 and 3.

#### DISCUSSION.

From the first experiment it is seen that relatively small doses of rat sarcoma or embryo skin are sufficient to induce in guinea pigs an antibody reaction such that the plasma of the treated animals becomes a poor medium for the growth of either sarcoma or skin cells. There was no evidence of even a relatively specific action on the part of the cytotoxins formed, the plasma from each animal exerting an equal inhibitory action on each kind of tissue. The plasma from the animal immunized with sarcoma proved to be slightly more active against each tissue than that obtained from the guinea pig immunized with embryo.

In the second experiment in which larger doses of tissue were used for immunization a more marked toxic action was demonstrable. Again no evidence of specificity was seen. As in experiment 1 plasma from one of the animals was found to be more active against both tissues. In this case, however, it proved to be that

obtained from the guinea pig immunized with embryo. It seems clear then that these differences in toxicity are to be referred to individual differences in guinea pigs in their reacting power and not to differences in the antigenic properties of the two tissues.

The third experiment shows that a still stronger cytotoxic serum may be obtained by a repetition of large doses of either tissue. Here again no evidence of specificity was found.

The fourth experiment shows that blood may be used to immunize against both sarcoma and skin, although the cytotoxic power of such a serum seems to be not so great as when either of the other tissues is used.

A second parallel set of experiments was carried out with two chick tissues, heart and intestine, for the production of cytotoxic sera in guinea pigs. The tissues were obtained from sixteen to twenty day chick embryos. These two tissues were selected because they exhibit characteristic types of growth in tissue cultures. From the heart a radial growth of connective tissue cells is regularly seen, while from pieces of intestine wide sheets of epithelial cells stretch out. Chick tissues grow fairly well in normal guinea pig plasma. In plasma from immunized guinea pigs the results were practically the same as those obtained with rat tissues; that is, no evidence of specificity was found. The experiments are therefore not given in detail.

#### SUMMARY.

1. The plasma of guinea pigs treated by injections of rat sarcoma exhibits a toxic action in tissue culture preparations on the cells of both rat sarcoma and rat embryo skin. Similarly, the plasma of guinea pigs immunized by injections of rat embryo skin is toxic for cells of both types.
2. Injection of rat blood immunizes against both sarcoma and embryo skin, although not so strongly as injections of the two tissues.
3. Guinea pigs receiving injections of either chick embryo heart or intestine develop cytotoxic substances for both of these tissues.
4. The preceding findings tend to show that cytotoxins formed after the injection of different body tissues into a foreign species are to no extent specific for the tissues injected.

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# AN EXPERIMENTAL STUDY OF THE HISTOGENESIS OF THE MILIARY TUBERCLE IN VITALLY STAINED RABBITS.\*

By HERBERT M. EVANS, M.D., FRED B. BOWMAN, M.D., AND  
M. C. WINTERNITZ, M.D.

(From the Anatomical and Pathological Laboratories of Johns Hopkins  
University, Baltimore.)

PLATES 36 TO 39.

## INTRODUCTION.

The inadequacy of the methods hitherto applied to the study of the histogenesis of the tubercle led us to produce tuberculosis experimentally in vitally stained rabbits. In rabbits injected with trypan blue the Kupffer cells stain deeply, while the blood cells are free from stain, and we thought that this might throw some light on the part played by the Kupffer cells in the formation of the miliary tubercle in the liver.

## HISTORICAL.<sup>1</sup>

Opinion is still divided as to the origin and structure of the miliary tubercle. About fifty years have elapsed since Virchow first demonstrated the tubercle, named it, and showed that it was the distinctive product of tuberculosis. Although Virchow saw little in its morphology save round cells, it was not long before Langhans found the giant cell and noted its practically constant occurrence. Schueppel discriminated the three cell types (lymphocytes, epithelioid cells, and giant cells) which we recognize to-day as being usually present, if not of invariable occurrence, and of valuable diagnostic aid. The typical arrangement or succession of these three cell types has come to be a law (Ziegler).

Though we are now aware that they are by no means pathognomonic for the tubercle, nevertheless the giant and epithelioid cells are peculiar structures, identical with none of the familiar fixed cells of the body, although descended from them. Their manner of descent and transformation have consequently claimed

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<sup>1</sup> The literature has been reviewed by Wechsberg, Dürck, Dürck and Oberdorfer, and others, and only the more important views will therefore be given.

much interest. After the discovery by Koch in 1884 of the etiological agent of tuberculosis, many investigators began to study the formation of the tubercle after the injection of pure cultures. One view identified with the name of Metchnikoff,<sup>2</sup> the other with that of Baumgarten, might be said to represent the views of the French and German schools respectively. Metchnikoff maintains that the tubercle is of leucocytic origin, and Baumgarten believes that the fixed tissue cells alone are responsible for its formation.

Metchnikoff's contention must be regarded as the natural outcome of his theory of the cells concerned everywhere in the defense of the body against bacterial disease. To the chief cells he has applied the term macrophage, meaning thereby a class of large mononuclear cells doubtless identified by others as wandering cells, which Metchnikoff and his followers considered to have arisen from the overgrowth of true lymphocytes. Stages in this transformation were described, so that there was no difficulty in imagining that with the increase in cell body, the deeply-staining lymphocytic nucleus became the large clear nucleus, poor in chromatin, of the epithelioid or giant cell. The phagocytic properties of the latter, however, were the chief cause of Metchnikoff's derivation of them from his macrophage, and he emphasized the almost invariable existence of bacilli in these cells. Unquestionably the convictions of Metchnikoff came more as a general result of his notions of the cells concerned in immunity than as the result of an exhaustive morphological research, but it is interesting to find that Koch himself was disposed to regard wandering cells as the chief elements in the tubercle, even though he imagined that their subsequent death might result in the participation of various fixed cells.

Baumgarten, and many investigators after him, have established the fact that the tubercle is not merely a heaping up of preformed elements, but that active proliferative processes are always concerned in its formation. The evidence for this was clear, for frequent mitoses are found, and inasmuch as these affect the chief cells of the immediate neighborhood, regardless of where the tubercle lies, Baumgarten and others have concluded that these cells alone are essential in tubercle formation, and that the most various tissue elements, including those as far apart as connective tissue and epithelium, could play this part, but that leucocytes could not. Indeed Baumgarten denied any but the latest and most trivial participation of leucocytes in the actual beginning of the tubercle, though subsequent investigators among his own countrymen could not, of course, confirm this. The preliminary outpouring of polymorphous cells and secondary lymphocytic infiltration which the French school had so clearly seen were undeniable, though Baumgarten's adherents denied a wider interpretation of this phenomenon as completely as the French school had denied the interpretation put on mitoses, the presence of which they also admitted. According to Baumgarten's theory, Kostenitsch and Wolkow describe the inception of a tubercle as follows: (1) the formation of a serofibrinous exudate, (2) migration of polynuclear leucocytes which rapidly disintegrate, (3) proliferation of fixed tissue cells to form epithelioid cells, (4) migration of mononuclear leucocytes which take a peripheral

<sup>2</sup> Metchnikoff's views are upheld by Yersin, Borrel, Leray, and Wallgren; Baumgarten's by Klebs, Kockel, Kostenitsch and Wolkow, Wechsberg, Miller, Watanabe, Oppenheimer, Straus, Morel, and others.

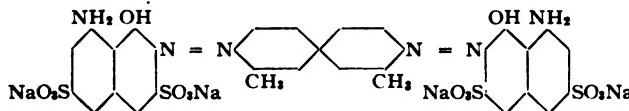
position around the epithelioid cells, and (5) secondary migration of polynuclear elements when the tubercle degenerates.

The question of the origin of the cells forming the tubercle depends on our ability to distinguish sharply cell types. Few reliable criteria have been advanced for such a discrimination, although the recent work of Wallgren and Oppenheimer should be mentioned. These investigators have taken advantage with us of the fact that the production of tubercles within the liver lobule permits us to work in a relatively simple territory, where besides the blood cells and endothelium of the hepatic sinuses, only the liver cells occur, all the connective tissue elements, for instance, being absent.

Oppenheimer, starting from the fact that silver particles are phagocytized largely by Kupffer cells, has produced hepatic tubercles after collargol injections. The tubercles which appeared after the transitory leucocytosis were composed of silver-containing epithelioid and giant cells, and Oppenheimer naturally derived these from the endothelium. It is unfortunate that the conclusiveness of his experiments is open to doubt on the grounds that normally some of the blood cells engulf the silver particles. Furthermore, Wallgren, also working with rabbit liver, has reached an opposite conclusion, and substantiates fully the lymphocytic origin of the cells. There appears to be sufficient reason, therefore, for a reinvestigation of the relatively simple liver tubercle with a more decisive method of study.

#### MATERIAL AND METHODS.

In our experiments rabbits were used exclusively. The animals were vitally stained by repeated intravenous injection of a freshly prepared aqueous solution of trypan blue. The dye is a benzidin of the following formula,



and has been used as a vital stain, by Bouffard, Goldmann, Schulemann, Evans, and others. The staining in the experiments to be recorded may be divided into two groups: the acute staining, where daily intravenous injections of twenty cubic centimeters of 1 per cent. solution of the dye were given during a few days, and which resulted chiefly in a pigmentation of the Kupffer cells; and the chronic staining where the injections were made at longer intervals and over a longer period of time. In these animals, besides the staining of the Kupffer cells, the blood of the liver capillaries contains many vitally stained macrophages. Both types of staining are adaptable to the study of the histogenesis of the tubercle, and the method of staining will be briefly given in each experiment.

Emulsions of bovine tubercle bacilli were made in the usual manner. The bacteria were carefully removed from the glycerin bouillon medium, dried on sterile filter paper, ground, and taken up in normal salt solution. This was then put into a shaking machine for two hours. The strength of the suspension was graded so that each cubic centimeter contained one milligram of dried tubercle bacilli.

The animals were anesthetized, a loop of bowel was exposed aseptically, and ten cubic centimeters of the suspension of tubercle bacilli were injected into a small radical of the mesenteric vein. The animals were killed at varying intervals, from one half hour to eleven days, and immediately injected through the ascending aorta by pressure, with a 10 per cent. solution of formalin. Under these conditions the tissues are rapidly fixed throughout, the intra-acinar capillaries are dilated, and most of their blood content is washed out. The liver was then removed, cut into small strips a few millimeters in thickness, and replaced in formalin. Satisfactory sections were obtained from frozen sections, and these were used throughout the study. With the above fixation, sections five micra in thickness were readily obtained. The sections were stained with aqueous cochineal, which contrasted well with the blue vital stain. Other sections were stained in Ziehl-Nielson carbol fuchsin for five minutes, decolorized in 10 per cent. hydrochloric acid alcohol, thoroughly washed in distilled water, and counterstained with Delafévier's hematoxylin. Weigert's fibrin stain was used in some of the early stages.

#### THE RESULT OF THE INJECTION OF VITAL STAIN UPON THE LIVER OF NORMAL ANIMALS.

The literature on the histology of the normal rabbit's liver has been reviewed recently by Wallgren. Concerning the Kupffer cells, Wallgren quotes Schilling who considers them to be endothelial phagocytes and functional stages of liver endothelium. He believes that they arise from normal liver endothelium, by swelling and paling of their nuclei, and that probably every endothelial cell passes through this change from time to time.

In order to check Schilling's results, we killed an animal and

ected formalin through the aorta. The liver cells occasionally contain two nuclei, and a mitosis may occasionally be found. The nuclei of the endothelial cells of the capillaries are relatively wide apart, and two are found opposite each other only in rare cases. They are closely approximated to the columns of liver cells, and vary considerably in appearance. Many of them are flat, like ordinary endothelium, and there are transition forms to the larger ones with vesicular nuclei that bulge into the lumen of the capillary. The larger ones rarely contain an ingested polynuclear leucocyte or granule of brown pigment.

#### EXPERIMENTAL PART.

##### VITALLY STAINED NORMAL ANIMALS.

*Rabbit 1.*—Mar. 14, 1912, 9 A. M. Injected with 20 c.c. of a 1 per cent. aqueous solution of dye in ear vein.

Mar. 15, 9 A. M. Killed. Fixed with formalin through aorta.

*Liver.*—The liver cells contain a few granules of trypan blue. The Kupffer cells are about normal in size and occurrence. Most of them contain a varying number of small blue granules. Endothelial cells with two nuclei or cells in mitosis are not found. Phagocytosis of polynuclear leucocytes or red blood cells is uncommon.

*Blood.*—The usual white blood cells, including lymphocytes and mononuclear and polynuclear leucocytes, occur and are unstained. Besides these large cells with clear non-granular protoplasm, vesicular nuclei and definite nucleoli occur. The nucleus may be round, oval, or bent. The nature of the cells is not always determinable. They appear to be similar to the large lymphocyte or polyblast emphasized by Wallgren.

*Interstitial Tissue.*—Normal; no vitally stained cells.

*Rabbit 97 A.*—Mar. 10 and 11. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of forty-eight hours.

*Liver.*—The cells contain a few small granules of vital stain. The Kupffer cells are more numerous, and many of them are increased in size. The majority contain blue granules of irregular sizes. These may be dense, or there may be only a few granules scattered near the nucleus, or even towards the tip of one of the protoplasmic processes of the cell. The dye is more abundant in the larger cells though the flat ones may contain a few granules. As the cell becomes larger, the nucleus becomes more vesicular, and rarely two nuclei may be found within one cell body. One mitotic figure was found. Phagocytosis occurs as usual.

*Blood.*—There are no vitally stained cells in the capillaries. The blood picture is similar to that of rabbit 1.

*Interstitial Tissue.*—An occasional clump of blue granules is found in the periportal tissue; but whether these are contained within the endothelium of the vessels or in the wandering cells cannot be determined.

*Rabbit 200.*—April 13, 14, 15, and 16. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of the fourth day.

*Liver.*—The cells contain definite, large, bright blue granules of dye, and have two nuclei. The Kupffer cells are conspicuous. They are definitely increased in numbers and most of them are large and heavily laden with vary sized granules of dye. Some of the enlarged Kupffer cells are no longer fixed attached to the liver columns, and may be free in the lumen with their processes extending to the column of the liver cells. These cells may be cut in such a way as to appear round and free in the capillary. Not only are the Kupffer cells enlarged, but an occasional one with two or three nuclei and a rare mitotic figure may occur. In the latter the pigment granules are never found in a spindle. The nuclei of the large Kupffer cells may be irregular, and assumed polymorphous shape. The phagocytic property of the large Kupffer cells is demonstrated by the frequent finding of polymorphonuclear leucocytes or red blood cells within their bodies.

*Blood.*—The injection of formalin through the aorta was unsuccessful, as a consequence, considerable blood is contained within the vessels. The larger veins contain normal unpigmented blood elements. The larger polyblast-like cells occasionally contain a few very fine granules of dye.

*Interstitial Tissue.*—There is an occasional pigmented cell, the nature of which could not be definitely determined.

*Rabbit 80.*—Six successive doses of 20 c.c. of 1 per cent. solution of trypan blue were given on six successive days. The animal died shortly after the last injection, i. e., early on the sixth day. The findings were similar to those in rabbit 200, except that two large pigmented giant cells were found in the liver.

*Rabbit 49.*—Nov. 23, 27, Dec. 6, 31, Jan. 5 and 13. 103 c.c. of 1 per cent. aqueous solution of trypan blue were given in approximately 20 c.c. doses. Jan. 19. Animal killed by a blow on the head, and 500 c.c. of 10 per cent. formalin injected through the aorta at a pressure of 80 mm. of mercury.

*Liver.*—The cells show a few granules of dye. Cells with two nuclei and occasional mitoses in the liver cells may be found. The Kupffer cells are conspicuous on account of their increased size and number, and their content of pigment granules. Kupffer cells with two or more nuclei are common and may be large giant cells with innumerable nuclei. Frequently they occupy the position of the normal Kupffer cells, but sometimes they appear to be free. Mitosis is never demonstrable in them, but all transitions occur from those with two, three, and even more nuclei. The nuclei of the cells are large and twisted so that they have a polymorphous appearance, and not infrequently the lobes may be connected by delicate threads of nuclear material. Phagocytosis is as usual.

*Blood.*—Besides the elements found in the previous cases, occasional large macrophages occur, often so densely pigmented that the nature of the cell is entirely obscured.

*Interstitial Tissue.*—The same as in the previous cases.

From the foregoing experiments it is evident that following successive intravenous injections of trypan blue the Kupffer cells become laden with the dye, enlarge, and increase in number, and finally form large free syncytial masses or giant cells containing one

several nuclei. The endothelial cells which undergo this transformation and finally become large free macrophages are filled with increasing numbers of the vital blue granules in their cytoplasm. The blood cells, on the contrary, remain free from the dye and undergo no noticeable changes. It is important to note that whereas the dye thus stimulates the formation of free endothelial cells, it does so only after an interval of four or five days; within this interval only mild proliferative changes occur, resulting merely in the enlargement of Kupffer cells and their multiplication *in situ* in the vessel wall. We shall find in tuberculosis a far more rapidly changing picture.

#### EXPERIMENTAL TUBERCULOSIS IN UNSTAINED RABBITS.

*Rabbit 101.*—Killed forty-eight hours after inoculation with a suspension of 10 mg. of bovine tubercle bacilli in superior mesenteric vein. Dense cellular masses are scattered through the liver, either in the region of the periportal vessels or in the intra-acinar capillaries.

*Liver.*—The cells are coarsely granular and vacuolated, but otherwise present nothing of note. The Kupffer cells are more numerous than in the normal liver, and vary in size. Many of them are much larger, and as they increase in size the nuclei become more vesicular. The smaller, more slender Kupffer cells lie against the column of the liver cells like an endothelial membrane, while the larger ones seem to be less firmly attached and may be seen almost free in the lumina of the capillaries. Here their contour is easily made out. They are spindle- or winged-shaped, with long processes that often cross the bed of the capillary before they approximate themselves to the wall of the liver columns. When cut at different angles they often resemble polyblasts. They may contain polymorphonuclear leucocytes, which in many instances are well preserved and appear to be as healthy as those free in the capillaries. Around these ingested cells is a clear vacuolar space, which may cause an indentation in the nucleus where the latter comes in contact with the ingested cell. The ingested cell gradually undergoes disintegration. The chromatin may first disappear and leave a pale homogeneously staining protoplasm containing only a few chromatin specks, or the protoplasm may be digested or shrunken, leaving a small structureless mass of chromatin. Mitotic figures occur abundantly in the Kupffer cells, and Kupffer cells with two or three nuclei are found.

*Blood.*—The capillaries are dilated. Here and there in the smaller capillaries a few red blood cells or a polynuclear cell may be seen. Mononuclear cells also occur, some of which are small lymphocytes. By far the greater number, however, are irregular round or oval cells whose nuclei present no constant picture. They are apparently quite active, and while some have the chromatin arranged wheel-like, others are in division. Some of the cells are definitely Kupffer cells, since they are still attached by one or more processes to the columns of liver cells. Others are not in the capillaries, but occur as small nests of cells in

indentations between the liver cell and the endothelial lining. In one such area the Kupffer cell may be seen sending a process down between the liver cells. The larger clumps or plugs are in the periportal vessels, whose endothelium is swollen and partly desquamated. The lumen contains, besides these desquamated cells, one or more giant cells, polynuclear leucocytes, and mononuclear cells. One nodule, for instance, has a large, crescentic, multinucleated giant cell at one point. The nuclei of this cell form a horseshoe, and several small beaded bacteria are visible in the protoplasm occur. The remainder of the nodule is composed of numerous stellate spindle and oval cells with pale vesicular nuclei. Two are dividing. The vessel wall is not clearly distinguishable, and the cellular accumulation is present to a slight extent in the surrounding tissue. The giant cells, Kupffer cells, and spindle or oval cells contain the bacteria, which occur occasionally in polynuclear leucocytes also.

In some areas pictures occur which suggest the possibility that these giant cells may increase in size by fusion. Cells with nucleus and protoplasm similar to that of the giant cell are found in close apposition to each other.

*Interstitial Tissue.*—In the region of the periportal spaces the interstitial tissue is obscured on account of the frequent localization of the clumps in these areas, described above. Fibrin could not be demonstrated in the clumps.

*Rabbit 84.*—Killed seven days after inoculation.

*Liver.*—The section shows the liver tissue to be infiltrated with large nodules which tend to become confluent. The cells show nothing of note except atrophy where the columns are pressed apart by the intravascular nodules. Kupffer cells are numerous. Many of them are large and multinucleated. Mitosis is frequent and was found in one cell with three nuclei. Cells may be seen dividing with the line of division perpendicular to the long axis of the vessel. Some of the Kupffer cells are more or less rounded and indistinguishable from the polyblasts in the capillaries except by their processes.

*Blood.*—The cell picture resembles that found in the previous experiments. Polyblast-like cells are abundant and many show mitosis. They are also found in nests beneath the endothelium.

*Plugs.*—The smaller plugs have a central giant cell surrounded by Kupffer cells and large round cells. The larger nodules show central caseation with a peripheral zone composed of the above cell types.

Bacteria are abundant and occur exclusively within the bodies of cells. These giant cells and particularly the Kupffer cells contain the organisms.

*Summary.*—The bacteria at the end of forty-eight hours are found intracellular, and frequent degenerative forms are encountered. The polynuclear leucocytes rarely contain tubercle bacilli, while the giant cells, Kupffer cells, and mononuclear cells contain them in the order named.

The Kupffer cells apparently are active. Frequent mitoses are found in them and Kupffer cells with two or more nuclei occur. These may form giant cells which may either occupy their normal

position or lie free in the lumen of the capillary. The giant cells do not show mitoses, but their nuclei are tortuous and polymorphous suggesting multiplication by budding. Elsewhere, within the center of tubercles pictures are occasionally encountered which suggest the possible formation of giant cells, or rather their increase in size by fusion, but these pictures are rare and not convincing.

Besides polynuclear leucocytes and definite lymphocytes in the capillaries, other large white cells occur which are difficult to classify. These cells are round or oval with homogeneously staining protoplasm and varying types of nuclei. They frequently show division. They are probably the cells spoken of as polyblasts by Wallgren. Some of them have streamers that extend to a neighboring Kupffer cell; some seem to be attached to the Kupffer cells, but the majority are free in the capillaries. Cells exactly like these may be found singly, or in nests lying in hollows of the liver cells below the endothelium. Individual cells of such a nest may be in mitosis. The Kupffer cell overlying the nest may also be in mitosis. The similarity between the cells of the nests and the large unclassified cells in the liver capillaries on the one hand, and the similarity and anatomical relation of both types to the dividing young Kupffer cells on the other hand are striking.

The nodules are of two types; those occupying the larger portal venules, and those of the intra-acinar capillaries. The first, even at the end of seven days, contain polynuclear leucocytes, though they are much less numerous than at the end of forty-eight hours. Besides these, large giant cells, stellate cells resembling Kupffer cells, and large mononuclear cells, compose the clumps. The vessel wall may or may not be intact in any one nodule. Outside the vessel similar cells may sometimes be found. Fibrin is not demonstrable.

The smaller intra-acinar nodules afford the more satisfactory study, consisting as they do of giant cells, which may or may not preserve their normal relation to the lining of the capillaries. Often the giant cell is pushed out and surrounded on all sides by cells similar to Kupffer cells, and by large mononuclear cells which may show mitosis, and occasionally by lymphocytes. These nodules completely fill the containing vessel, and in the later stages the centers may be caseous.

## EXPERIMENTAL TUBERCULOSIS IN VITALLY STAINED RABBITS.

The difficulty of determining the origin of the cells of the young tubercle from ordinary stains is evident from the above description. It is impossible to say what the origin may be, beyond the fact that mononuclear cells are involved. Whether the endothelial or the mononuclear blood elements are concerned cannot be decided. The intermingling of the two cell types has taken place, and every transition from either type to the giant cell can be imagined. The vital stain, free from any possibility of inclusion of the blood cells, should furnish discriminating light here.

*Rabbit 98.<sup>a</sup>*—Killed twenty-four hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and one half hour after inoculation with tubercle bacilli.

*Liver*.—Aside from slight pigmentation, the cells show nothing abnormal. The Kupffer cells are enlarged and relatively deeply stained. They correspond in size and intensity of vital staining to those of rabbit 97 A. Mitotic figures are found in much greater frequency than in the animals that received vital stain alone, and Kupffer cells containing two to three or four nuclei, though rare, may be found. The Kupffer cells show phagocytosis.

*Blood*.—Except for an apparent increase of polynuclear leucocytes there is no change in the blood picture.

*Plugs*.—Both in the larger interlobular veins and in the intra-acinar capillaries cell accumulations occur. The walls of the veins may be broken and their fibres stained vitally. Within the vein a bluish fibrillar substance occurs, in which many polynuclear leucocytes, a few red blood cells, and an occasional lymphocyte are enmeshed. In the center of these areas clumps of bacteria are frequently found. The surrounding polynuclear cells seldom contain bacteria, but the Kupffer cells frequently contain organisms even in distant areas. These cells may show mitosis, and a small group of cells may accumulate around the bacteria. Fibrin is not demonstrable.

*Rabbit 100.*—Killed thirty hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and six hours after inoculation with tubercle bacilli. Clumps of blue staining material scattered throughout the liver are readily seen with the naked eye.

*Liver*.—The cells contain granules of trypan blue. Many of the Kupffer cells are enlarged. Granules of vital stain are found in them as well as in the normal endothelial cells, but a number of cells are still unstained. Kupffer cells with several distinct nuclei occur occasionally. These may be so large that they almost occlude the lumen of the capillary. Mitoses occur in the Kupffer cells, but are not demonstrable in those with more than one nucleus. Phagocytosis is marked.

*Blood*.—The capillaries contain little blood. Here and there a few red blood cells occur. Polynuclears may also be seen, but they are not abundant. So

<sup>a</sup> This animal was not injected with formalin after death on account of the risk of washing out the fresh capillary plugs in the liver.

of the mononuclear cells are definite lymphocytes, but others resemble polyblasts. None of the blood cells are pigmented with the vital stain.

**Plugs.**—Most of the plugs are small and lie in the intra-acinar capillaries which they may entirely occlude. They consist of large, swollen, pigmented Kupffer cells with varying numbers of large vesicular nuclei, a few polymorphonuclears, and an occasional mononuclear cell. The Kupffer cells are easily identified by their shape and pigment content. Some of them may be still attached to the vessel wall. They may contain bacteria, but this is difficult to demonstrate, since the bacteria are obscured by the vital stain. Here and there a mass of blue staining rods which resemble the bacteria occur. The larger plugs resemble those in the half hour stage.

*Rabbit 97 B.*—Mar. 10. 20 c.c. of trypan blue injected into ear vein.

Mar. 11. 20 c.c. of trypan blue injected in ear vein and inoculated with tubercle bacilli.

Mar. 12. Killed twenty-four hours after inoculation.

Scattered through the liver tissue are plugs of material conspicuous on account of their blue stain.

**Liver.**—The cells are normal in appearance and contain granules of trypan blue. The Kupffer cells are increased in size and number and contain many blue granules. The number of granules varies; some have only a few small ones, while others are so densely packed with them that the nucleus is obscured. Mitotic figures occur (figures 1, 2, and 3), and multinucleated cells are much more abundant than in the six hour stage. Phagocytosis is marked.

**Blood.**—The capillaries are dilated, but contain little blood. Polynuclears and red blood cells occur as usual. Mononuclears are more abundant. Most of them resemble polyblasts, but some are evidently Kupffer cells cut tangentially, and still have a process extending to the wall of the capillary. These have blue granules. Still others are found similar to them in every respect, but not attached to the vessel wall and without pigment granules. These cells are occasionally found lying in indentations of the liver cells below the Kupffer cells. With the exception of one polymorphonuclear leucocyte containing a few pigment granules, the cells of the circulating blood are normal.

**Plugs.**—These stand out sharply on account of the concentration of vital stain in them. Some of them consist of a single giant cell which is sometimes still attached to the vessel wall, and surrounded by only a few stellate Kupffer cells well laden with pigment granules. The larger plugs show a center of bluish material, in which polynuclear leucocytes and mononuclear cells are found. Surrounding these one or more pigmented Kupffer cells, or mononuclear, polyblast-like cells occur and form the remaining constituents of the plugs.

**Bacteria.**—A few bacteria are found in the Kupffer cells and in the giant cells. They are, however, made out with difficulty, as they are either stained or overshadowed by the trypan blue.

*Rabbit 92.*—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue. Inoculated with tubercle bacilli two hours later.

Mar. 3. Intravenous injection of 21 c.c. of trypan blue. Animal killed thirty-six hours after inoculation.

**Liver.**—The section is studded with many masses of vitally stained cells,

usually near the periportal spaces. The liver cells are coarsely granular vacuolated. They contain a few small granules of trypan blue. The Kupffer cells are almost all enlarged and appear more numerous. The greater number of them are stained as usual.

A considerable number of cells with two, three, or more nuclei are found. These may still occupy their positions on the capillary lining. Giant cell formation is particularly marked (figures 5, 6, and 7). Mitoses are found in unusually large numbers in Kupffer cells with one nucleus, but not in multinucleated cells.

*Blood.*—The capillaries contain red blood cells, and polynuclear leucocytes but the mononuclear cells predominate. Some of them are lymphocytes, but the majority are similar to polyblasts. The latter are not infrequently seen in mitosis.

*Plugs.*—More numerous than in rabbit 97 B. In the larger ones, the polymorphonuclear leucocytes have to a great extent disappeared. Deeply pigmented giant cells still partially attached to the vessel wall, stellate pigmented and non-pigmented Kupffer cells, and polyblast-like cells occur as usual. The polyblast-like cells in some places seem to push the giant cell away from its attachment to the vascular wall and infiltrate the surrounding tissue to a slight extent. Mitoses occur in the polyblast-like cells and are often demonstrable in the Kupffer cells in the vicinity of the larger plugs. The smaller intra-acinar plugs present the usual picture. They invariably contain a large pigmented multinucleated giant Kupffer cell (figure 8), and in some instances are surrounded only by irregularly pigmented Kupffer cells. The Kupffer cells vary in the amount of their pigment. In other instances polyblast-like cells are associated with the Kupffer cells at the periphery of the giant cells. These cells are not pigmented but sometimes cells almost identical in type contain a few granules. The smaller plugs may entirely occlude the capillary, and in some instances, where the giant cell is still attached in its original position, the lumen of the vessel may be entirely obstructed by it alone.

*Bacilli.*—Acid-fast involution forms occur rarely in the giant and Kupffer cells, but they are demonstrated with difficulty.

*Rabbits 95 and 96.*—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue, and 10 mg. of tubercle bacilli.

Mar. 3. Intravenous injection of 20 c.c. of trypan blue.

Mar. 4. Killed fifty-four hours after inoculation.

A detailed description of the cases will be omitted. Only the points will be emphasized which have not been noted in the previous experiments.

*Liver.*—The Kupffer cells show the usual pigmentation, mitosis, phagocytosis and giant cell formation. One multinucleated Kupffer cell was found in which one of nuclei was in mitosis.

*Blood.*—The usual cellular elements are present. Polyblastic cells are most conspicuous. They occasionally show mitosis, and some of them contain a few minute pigment granules. Cells similar to these, but more pigmented, occur singly or in nests in small pockets formed between the Kupffer cells and the liver cells. Individual cells in the nests may be undergoing mitosis. The direct origin from Kupffer cells was not demonstrable, although large Kupffer cells in their vicinity, in some cases lying over the nest, were seen to be undergoing mitosis.

*Plugs.*—The larger plugs contain fewer polynuclear leucocytes; the smaller

ones are more cellular, otherwise they resemble those described in the previous experiment. In rare instances pigmented Kupffer cells may be so closely packed against a giant cell that the possibility is suggested of a giant cell increasing in size by fusion with Kupffer cells. In one tubercle occurring in a large periportal vein a tubular structure was found lined by swollen, pigmented Kupffer cells; at one end the cells dilated and filled the lumen, giving the appearance of a giant cell.

*Rabbit 74.*—Jan. 16. Intravenous injection of 14 c.c. of trypan blue.

Jan. 17. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 18. Intravenous injection of 20.5 c.c. of trypan blue and 10 mg. of tubercle bacilli.

Jan. 19. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 20. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 21. Killed seventy-two hours after inoculation with tubercle bacilli. The giant cells are particularly numerous and stained brilliantly with the vital stain. They are so often attached to the vessel wall that where this is not found it may be attributed to the plane of the section. One giant cell had three nuclei, one of which was undergoing mitosis. The polynuclear leucocytes in the circulating blood rarely contain a few blue granules. Kupffer cells are found free in the capillaries; they may retain their usual shape, or may be rounded with only a single process connecting them with the capillary lining. Such cells may be pigmented, and are hence distinguishable from the polyblastic cells.

*Plugs.*—In one of the plugs a non-pigmented giant cell was found. Pigmented and non-pigmented Kupffer cells, indistinguishable from polyblasts, may be found free in the capillaries. They may have several nuclei and the folding of the nucleus suggests an increase by budding. It is possible that these Kupffer cells are young, and the transition forms suggest that they may be the source of non-pigmented giant cells.

Experiments were carried on similar to those described in which the animals were killed at varying intervals up to eleven days. No new facts were brought out, however, and consequently a detailed description will not be given.

*Rabbit 44.*—A chronically stained animal had received the usual dose of trypan blue at regular intervals from Nov. 21 to Feb. 9, 160 c.c. of dye being injected during this period. On Feb. 15 the animal was inoculated with tubercle bacilli and on Feb. 19 a lobe of the liver was excised for study.

*Liver.*—The cells show the usual picture found in chronically stained animals. Large numbers of deeply stained macrophages occur throughout the liver capillaries. Definite tubercles were found in considerable numbers, usually located near the periportal areas. These are composed of central giant cells, and epithelioid and mononuclear cells. Occasional polynuclear leucocytes are found in the larger clumps. The point of chief interest, however, is that the tubercles contain practically no vital stain. The small amount of stain that is found in the tubercle appears as fine granules in the giant and epithelioid cells.

This case (rabbit 44) seems to us of interest from two points of view. First, the contention that preexisting wandering cells form the tubercle is decisively disproven. There existed in this animal a great number of large free wandering cells electively stained so that their participation in the tubercle would have given us stained cells there. Secondly, the experiment demonstrates that the vital dye must be present in quantity in the body fluids so as to be accessible to

the new growth at the time of its formation. All our studies have shown the electivity of the tubercle for the vital stain, yet in rabbit 44, where no free stain was available, the tubercles were almost colorless. That failure to stain the tubercles vitally was not due to their leucocytic nature or to any refractility to the vital dye was shown by the experiments begun as was rabbit 44, but terminated by the use of a vital dye of another color.<sup>4</sup> In these experiments red tubercles could be grown in blue livers.

**Summary.**—Within half an hour after the inoculation of tubercle bacilli in vitally stained animals cellular accumulations are found particularly in the region of the periportal vessels and in the intra-lobular capillaries. The tubercle bacilli are found in clumps free from the centers of the larger cellular masses, but even in the early stages a large number of organisms are found in the bodies of the cells. The polynuclear leucocytes contain relatively few bacteria, but many of the Kupffer cells, even in areas remote from the cellular accumulation, contain organisms. In the later stages the clumps of bacilli become less conspicuous, and organisms are demonstrable only in the bodies of Kupffer cells. The blue vital stain interferes somewhat with the demonstration of the organisms for it is very intense in the case of cells containing the bacilli. At the end of thirty minutes the plugs in the large vessels are partly composed of a homogeneous blue staining material in which are large numbers of polynuclear leucocytes. Occasional red blood cells and lymphocytes may be found here, but fibrin is not demonstrable. The vessel wall stains vitally, and this is probably the result of an injury brought about by the presence of the organism. The polynuclear leucocytes in the clumps gradually disappear and are replaced by cells which will be described below. At the end of half an hour mitotic figures are relatively frequent in the Kupffer cells, and this process continues through all the stages studied. As a result, Kupffer cells are found at the end of six hours with two or more nuclei, and at the end of twenty-four hours and later stages large brilliantly pigmented multinucleated giant cells lying in the position of the Kupffer cells form one of the most conspicuous elements in the section. The large Kupffer

<sup>4</sup> We employed dyes whose usefulness in this respect was discovered by Evans and Crowe (*Bull. Johns Hopkins Hosp.*, 1914 (in press)). Diamine fast scarlet 10 BF is a brilliant red which can be fixed in the tissues in formalin.

ells and giant cells<sup>5</sup> usually contain bacteria, and it is especially around these cells that the formation of the miliary tubercle may best be studied. The giant cell may lie partially or entirely free in the capillary connected only by a streamer to the lining cells. It is stained intensely vitally and, as a rule, it is surrounded by a varying number of cells which may all be stellate or wing-shaped Kupffer cells, containing more or less blue pigment. In this way a miliary tubercle may be found composed entirely of pigmented cells. Some of the Kupffer cells are not pigmented, in which case they are recognizable only by their peculiar form. This involves the separation of the non-pigmented cells from a third type of cell which closely resembles the polyblast. This cell is frequently found in the capillaries, and seems to occur in greater numbers after the six hour stage. Mitotic figures are found in them. They also occur singly, or in nests lying between the Kupffer cells and the liver columns. As a rule, they are not pigmented, but occasionally

<sup>5</sup> The tubercular giant cell noted by Rokitansky and Virchow, and emphasized by Langhans, Wagner, and Schueppel, has caused much discussion, particularly as to whether it is unicellular or multicellular in origin. Weigert, Straus, and Oppenheimer advocate the first theory; Kostenitsch and Wolkow, Kockel, Miller, and Watanabe consider that it results from a confluence of proliferated fixed tissue cells, and Metchnikoff, Borrel, and Wallgren think that it results from the fusion of white blood cells. Metchnikoff, however, has noted the formation of giant cells in the liver of *Spermophilus citillus* ("Ziesel") by a budding of the nucleus. Besides the true giant cells, Weigert, Arnold, Kochel, and others describe pseudogiant cells brought about by plugging of small vessels or bile ducts with exudate.

The nuclei of the large Kupffer cells are polymorphous and frequently several almost independent lobes may be found connected by slender strands of nuclear material. It seems probable, therefore, that the nuclei of the giant cells increase by direct division. Rarely pictures occur which suggest that the giant cells may increase in size by fusion with neighboring Kupffer cells. In the center of the larger tubercles deeply pigmented Kupffer cells closely approximated to the giant cell also suggest the possibility of this fusion. Consequently from the evidence that we have we should not deny the ability of more or less separate Kupffer cells to coalesce more perfectly to form the giant cells. But the endothelium of the hepatic capillaries is normally a common syncytial mass, and giant cell formation seems to us to be probably an intense unicellular response rather than an agmification phenomenon. A series of stages based on the unicellular hypothesis can be found in the growth of the young giant cell, although as a whole this question is of subsidiary interest compared with the manner of origin of the tissue.

a few fine granules of blue may be seen in the protoplasm. The cells occur abundantly in the larger tubercles.

Tuberculosis produced in chronically stained animals, in which no trypan blue was present in the circulation at the time of inoculation, resulted in the production of typical non-pigmented miliary tubercles. This is of interest because in chronically stained animals large numbers of deeply stained macrophages are always found in the circulation. These old macrophages are, however, seldom concerned in the young tubercles that arise.

#### SUMMARY AND CONCLUSION.

In our study of the histogenesis of the miliary tubercle developing inside the liver lobule in animals that have been stained vital while inoculated with bovine tuberculosis, the controls enable us to recognize the manner in which the vital stain affects the liver. There is therefore no possibility of confusing the effects due to the organism with the effects due to the dye. It is, however, of interest to note that the effects are closely related. The vital stain alone is able to produce gradually some of the same changes that occur with far greater rapidity in experimental tuberculosis. Although in a few hours the Kupffer cells of tuberculous animals begin to react to the disease, in the case of normal animals stained vital they do not do this until after the third or fourth dose of successive daily injections. After many days, nevertheless, the vital stain alone produces enlargement, proliferation, and separation of Kupffer cells so that these are converted into large free phagocytes which may possess one or several nuclei. These are the gigantic macrophages of chronically stained animals. In all our experiments we have used only acutely stained animals, so that the effects of the dye itself are never sufficient to produce the changes. In fact there is no evidence that the dye accentuates the changes appreciably during the time involved in the experiment. The dye, however, shows the type of the cells entering into the tuberculous granuloma, for when fed to the body fluids in abundance trypan blue finds its way into all cells capable of receiving it. The vital stain is, as it were, a physiological test for the cells. Whatever the fundamental nature

f the vital stain produced by trypan blue and the benzidine dyes may be, it is important that this reaction does not occur to any appreciable extent with mononuclear blood cells, and that it does occur emphatically in the case of the hepatic endothelium. By means of this vital test, then, the following phenomena occur when suspensions of tubercle bacilli are let into the portal blood stream. The organisms, swept on by the blood stream, finally lodge in the terminal branches of the portal vein, where they plug the vessels and continue to multiply. They injure the vessel wall and cause around them an exudative inflammatory process, and finally lead to the formation of tubercles situated not only in these areas but also within the liver lobule. The injury to the vessel wall is manifested in the early stages by the presence of vitally stained areas in its structure. The bacteria at the end of half an hour are found to be extracellular in clumps in the larger vessels, but already to some extent in the bodies of vitally stained Kupffer cells throughout the liver. Exudative inflammation manifests itself by the presence of a transitory accumulation of poly-nuclear leucocytes about the bacterial clumps, which may be seen as early as half an hour after the inoculation. They continue to be present in the larger cell clumps of the periportal areas for many days, but they are rapidly replaced by other cells, mononuclear in type, so that within a day the histological appearance of the portal plug has changed radically. The mononuclear cell thus entering most actively into the reaction is endothelial and not hematogenous in origin, the vital stain enabling us to make a clear distinction. This fact, evident in the portal plugs, is decisively shown in the case of tubercles developing within the liver lobule. Such tubercles probably result from the localization of individual organisms within the Kupffer cells, for the initial stages of such a probable cycle have been found by us. They consist of the occurrence of mitoses in certain Kupffer cells where the Ziehl-Nielson method shows a bacillus or several bacilli to have been phagocytized (figure 4). Rapid growth of the infected cell now takes place, and at thirty-six hours the multi-nucleated giant cell produced is largely separated from the other endothelium of the vessel wall. Many bacilli exist within the protoplasm of these cells (figure 5), which are especially distinguishable

by their intense reaction to the vital stain. They have received trypan blue to such an excess that low power views of liver sections at the thirty-six hour stage show these cells as deep blue strands (figure 8). The origin of the giant cell from the Kupffer cell is evident not only from the above sequence and from the elective staining but also from the fact that even when fully formed, protoplasmic strands still join it to its mother tissue,—the normal endothelium of the vessel. The strands entangle other cells in their meshes, especially mononuclear blood cells, one of which, of the polyblastic type, has homogeneous protoplasm and is not infrequently encountered in mitosis. These cells are unquestionably of importance in the lesion of tuberculosis. We have seen them abundantly in the capillaries soon after the inoculation and they also occur singly or in nests between the Kupffer cells and liver columns. They are, as a rule, free from the vital dye. They continue to be concerned in the further growth of the tubercle and with the connective tissue cells make the structure of older tubercles relatively complex. On the other hand, little complexity occurs in the structure of the young intralobular masses. The miliary tubercle formed at the end of thirty-six hours is composed of a giant cell, surrounded by epithelioid cells and by blood cells of the above polyblastic type. The giant cell and its so called epithelioid cells are selectively stained and are exclusively derived from the hepatic endothelium.

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## EXPLANATION OF PLATES.

## PLATE 36.

FIGS. 1, 2, and 3. Three stages in the mitosis of Kupffer cells from the liver of a rabbit twenty-four hours after inoculation with bovine tubercle bacilli. Large stellate endothelial cells are densely laden with the vital blue granules of trypan blue, 40 c.c. having been given intravenously in two doses. The sections are counterstained with aqueous cochineal.

## PLATE 37.

FIG. 4. Kupffer cell from the same rabbit as figures 1, 2, and 3. The section is stained by the Ziehl-Nielson method and shows the body of a Kupffer cell in mitosis containing three bacilli. The presence of these might be considered as the immediate stimulus for growth of the cell.

FIG. 5. Young endothelial giant cell from the liver of a rabbit thirty-six hours after inoculation with bovine tubercle bacilli, stained as in figure 4 by the Ziehl-Nielson method. The cell is still connected to the vessel wall by protoplasmic processes; within it many bacilli and vital blue granules are found; four nuclei are included in the section; mitosis of any of the nuclei in the giant cell is not seen, so that direct division probably occurs.

## PLATE 38.

FIGS. 6 and 7. Young endothelial giant cells from the liver of the same rabbit as figure 5. The counterstain is aqueous cochineal. The enormous engorgement of these cells with the vital blue is readily seen. As yet no epithelioid cells have appeared round the giant cells. The giant cell of figure 7 is already almost free, but that shown in figure 6 is still part of the endothelial wall, the nuclei of which have increased rapidly, so that a syncytial mass was formed.

## PLATE 39.

FIG. 8. Low power view of a thirty-six hour old tubercle, showing the remarkable increase of the vital stain in the tubercle cells as compared with the remaining tissue. The tubercle is still almost purely the central giant cell, though several future epithelioid cells are noticeable.



FIG. 1.



FIG. 2.



FIG. 3.

(Evans, Bowman, and Winternitz: Miliary Tubercl in Rabbits.)





FIG. 4.

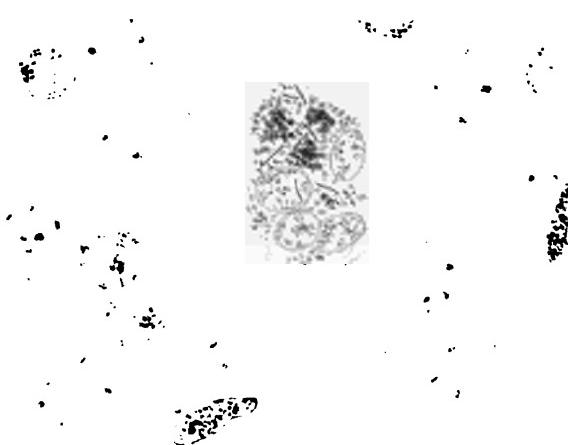


FIG. 5.

(Evans, Bowman, and Winternitz: Miliary Tuberclie in Rabbits.)



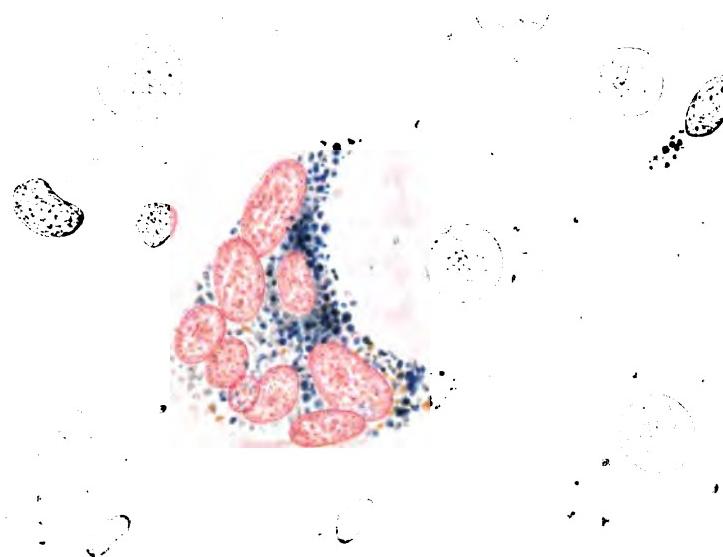


FIG. 6.

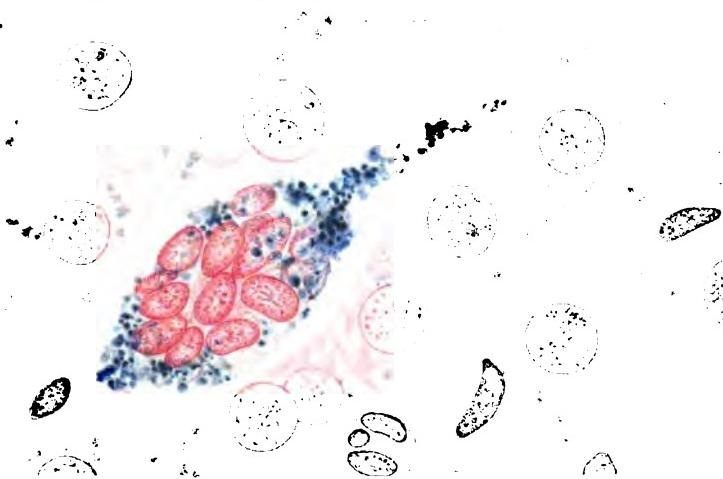


FIG. 7.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)



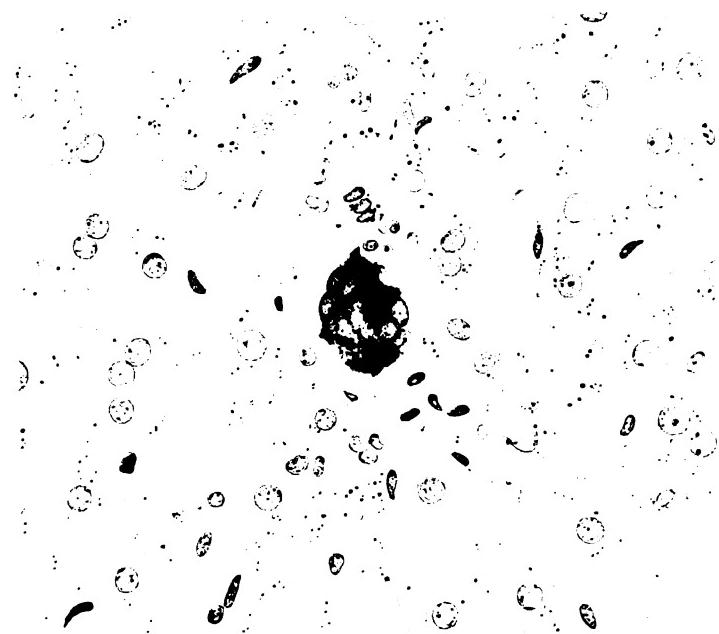


FIG. 8.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)



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# THE INDOPHENOLOXYDASE CONTENT OF TISSUES FROM RABBITS INFECTED WITH PNEUMOCOCCUS.\*

By FLORENTIN MEDIGRECEANU, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The property of animal tissues to oxidize a mixture of naphthol and paraphenylenediamine into indophenol was first demonstrated by Römann and Spitzer.<sup>1</sup> Since then it has frequently been used as a test for oxydases, both in plants and animals. Recently Vernon<sup>2</sup> devised a method for using this reaction quantitatively and has applied it to a number of problems, such as the quantitative distribution of the indophenoxydase in various animal tissues, the relation of its action to lipoids, etc. According to Battelli and Stern,<sup>3</sup> indophenoxydase belongs to the group of oxidizing agents which probably play an important part in the respiratory processes in the tissues. They call these oxidizing agents oxydones. The oxydones are not washed away from the tissues by water; alcohol and trypsin destroy them easily; the maximum activity occurs at 40° C. It seemed of interest to study the behavior of the indophenoxydase of animal tissues during pneumococcus infections. The problem has a bearing on the changes in the oxidation processes occurring in infectious diseases generally. In the present paper are recorded comparative figures showing the indophenoxydase content of tissues from rabbits that died of general pneumococcus infection and of tissues from normal control animals.

## METHOD.

The rabbits were given intravenously 0.2 to 0.5 of a cubic centi-

\* Received for publication, January 15, 1914.

<sup>1</sup> Römann, F., and Spitzer, W., *Ber. d. deutsch. chem. Gesellsch.*, 1895, xxviii,

<sup>2</sup> Vernon, H. M., *Jour. Physiol.*, 1911, xlvi, 402; 1911-12, xlvi, 96; 1912-13, xlvi, 374.

<sup>3</sup> Battelli, F., and Stern, L., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 212.

meter of a freshly prepared virulent pneumococcus broth culture. Death followed usually after sixteen to twenty-two hours. The normal control animals were killed by a blow on the neck at the same time that the infected rabbits died, or within one to two hours later. The organs from both the infected and the normal animals were taken out, prepared, and kept under the same conditions.

The estimation of the indophenoloxidase content was made according to Vernon's<sup>4</sup> method. Five cubic centimeters of the reagent mixture, containing in 100 cubic centimeters a solution of M/1,5 α naphthol and paraphenylenediamine and M/620 sodium carbonate, are exposed to the action of 0.5 of a gram of the finely chopped tissue in Petri dishes, 8.8 centimeters in diameter, with frequent repeated stirring. The reagent solutions are mixed immediately before their addition to the minced tissue, and for each test a fresh pipette is used.

The time of exposure was one half hour for the renal cortex and the heart muscle, and one hour for the liver tissue. It was found that this period of time is sufficient to obtain definite information in regard to the activity of the indophenoloxidase present.

The indophenol formed is then extracted with ten cubic centimeters of 96 per cent. alcohol for one half hour, filtered, and the quantity calorimetrically estimated by diluting a certain number of cubic centimeters of it with 50 per cent. alcohol to the depth of color of the standard solution. Test-tubes of the same diameter and thickness must be used. The standard solution is prepared by diluting 1.4 cubic centimeters of the above mentioned reagent mixture to 200 cubic centimeters with 50 per cent. alcohol and keeping it there for four days, or until the maximum of color is reached. It contains 0.01 of a gram of indophenol in 100 cubic centimeters. The standard solution is renewed every week.

A large number of experiments have been made under various conditions. The more constant and reliable results have been obtained with liver, renal cortex, and heart muscle. They are recorded in the following tables, where other details of the experiments are also given.

<sup>4</sup> Vernon, H. M., *loc. cit.*

TABLE I.<sup>5</sup>

*Pneumococcus Rabbit Died 22 Hours after the Intravenous Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed at the Same Time. Percentage of Indophenol Formed.*

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
Tissues kept in ice chest						
13.4 <sup>6</sup>	6.0	11.5	7.9	17.5	15.7	10 to 11 hrs. after death.
9.1	<3.0	10.6	7.2	17.5	12.1	30 to 31 hrs. after death.
12.1	<3.0	12.7	6.0	—	—	3 days later.
Tissues autolyzed at 37° C. <sup>7</sup>						
12.1	4.0	10.3	7.2	17.0	15.7	10 to 11 hrs. after death. Autolysis at 37° C. lasted for 1 hr.
9.1	<3.0	8.4	<3.0	12.7	12.1	30 to 31 hrs. after death. Autolysis at 37° C. of the liver and kidney tissues lasted for 2½ hrs., of the heart muscle for 5 hrs.

TABLE II.

*Pneumococcus Rabbit Died 22 Hours after Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed 1 Hour Later. Percentage of Indophenol Formed.*

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
Tissues kept in ice chest						
13.4	6.0	11.5	7.9	17.5	15.7	10 hrs. after death.
9.1	<3.0	10.6	7.2	17.5	15.7	30 hrs. after death.
6.6	<3.0	11.5	5.4	—	—	15 dys. later.
Tissues autolyzed at 37° C.						
12.1	4.0	10.3	7.2	17.0	15.7	10 hrs. after death. Autolysis at 37° C. lasted for 1 hr.
9.1	<3.0	8.4	<3.0	12.7	12.1	30 hrs. after death. Autolysis at 37° C. of the liver and kidney tissues lasted for 2½ hrs., of the heart muscle for 5 hrs.
6.0	<3.0	9.0	<3.0	—	—	15 dys. later. The tissues have been kept in the ice chest since the last experiment.

<sup>5</sup> The temperature at which the figures recorded in these tables were obtained varied between 22° and 28° C. Each normal control experiment was made at the same time and under the same conditions as the pathological.

<sup>6</sup> The figures that follow are not corrected for the spontaneous oxidation of the reagents into indophenol. On account of the short duration of the experiments (one half to one hour) the quantity formed is negligible.

<sup>7</sup> The autolysis at 37° C. was carried out in small Petri dishes 4 cm. in diameter, hermetically sealed with vaselin, in order to prevent the evaporation of the tissue water.

*Content of Tissues from Rabbits.*

TABLE III.

*Two Rabbits Have Been Inoculated Intravenously with Pneumococcus Broth Culture. Death Followed after 40 and 41 Hours Respectively. Normal Control Rabbit Was Killed 1 Hour Later.*  
*Percentage of Indophenol Formed.*

Renal cortex.			Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
15.7	15.0	15.0	15.7	12.0	22 and 24 hrs. after death. Tissues kept in ice chest.
9.1	<3.0	<3.0	4.2	<3.0	22 and 24 hrs. after death. Autolyzed at 37° C., kidney tissue for 7 hrs., heart muscle for 19 hrs.

TABLE IV.

*Pneumococcus Rabbit Died 16 Hours after Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed 2 Hours Later.*  
*Percentage of Indophenol Formed.*

Liver.			Renal cortex.			Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture. <sup>8</sup>	Pneumococcus rabbit.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
9.1	—	10.9	10.9	—	9.6	13.9	12.0	10 to 12 hrs. after death. Tissues kept in ice chest.
<3.0	<3.0	<3.0	9.1	9.1	4.2	10.2	7.2	10 to 12 hrs. after death. Tissues autolyzed at 37° C., liver and kidney for 3 hrs., heart muscle for 9 hrs.

TABLE V.

*Normal Rabbit. All Tissues Were Kept at Room Temperature, 23° to 28° C.*  
*Percentage of Indophenol Formed.*

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
15.0	15.0	10.8	10.6	18.6	18.6	6 hrs. after death.
10.6	10.6	7.0	7.1	13.8	17.4	20 hrs. after death.
9.6	8.0	7.0	6.0	—	—	44 hrs. after death.
<3.0	<3.0	<3.0	<3.0	9.0	9.0	6 dys. after death.

<sup>8</sup> One drop of a freshly prepared pneumococcus broth culture was added to the normal tissue in all similar experiments.

TABLE VI.  
*Normal Rabbit.*  
*Percentage of Indophenol Formed.*

Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
14.1	—	27.0	—	6 hrs. after death. Kept in ice chest.
10.5	12.0	14.0	13.5	6 hrs. after death. Autolyzed at 37° C. for 3 hrs.

TABLE VII.  
*Normal Rabbit.*  
*Percentage of Indophenol Formed.*

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
Tissues kept in ice chest						
17.7	—	10.3	—	—	—	8 hrs. after death.
18.0	—	10.3	—	22.7	—	14 hrs. later.
19.0	—	10.5	—	19.0	—	5 dys. later.
62.0	7.0	9.0	9.0	19.3	10.3	8 hrs. after death. Autolyzed at 37° C. for 2 hrs.
—	—	8.0	8.5	12.7	11.3	14 hrs. later. Autolyzed at 37° C. for 4 hrs.

#### SUMMARY AND CONCLUSIONS.

1. The action of indophenoloxidase is generally diminished in the tissues (liver, renal cortex, heart muscle) of rabbits that die of pneumococcus septicemia. The diminution is more frequent and marked in the liver and kidney than in the heart muscle.

2. The diminished activity of indophenoloxidase becomes more evident when the tissues undergo autolysis. At 37° C. this ferment is much more easily destroyed in the tissues of the infected animals than in those from normal control animals (tables I, II, III, and IV). Exceptions are rare and they occur chiefly with the heart muscle.

3. Normal rabbit tissues, inoculated *in vitro* with pneumococcus culture, do not lose their indophenoloxodase much more quickly than do the normal controls, when they are kept either at room temperature or at 37° C. (tables IV, V, VI, and VII).

4. It is therefore probable that the diminished activity of the indophenoloxodase of tissues from rabbits with pneumococcus septicemia is not due to the presence of the pneumococcus in these tissues but that it is associated with a pathological change in the animal during life.

A COMPARATIVE STUDY OF THE RATE OF PROTEOLYSIS OF TISSUES OBTAINED FROM RABBITS INFECTED WITH PNEUMOCOCCI AND OF TISSUES FROM NORMAL RABBITS.\*

By FLORENTIN MEDIGRECEANU, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

In the previous paper<sup>1</sup> it has been shown that the activity of indophenoloxydase undergoes a diminution in the parenchymatous tissues, especially the liver and kidney, of rabbits that died of a general infection with pneumococci. This ferment is more quickly destroyed during autolytic processes in the organs from rabbits infected with pneumococci than in those from normal control animals. The presence of the pneumococcus was excluded as being responsible for this difference. It was thought that a more rapid breaking down of the nitrogenous bodies, the proteins, might be associated with the increased lability of the indophenoloxydase in the tissues of the infected rabbits.

In order to obtain definite information on the rate of proteolysis in the tissues studied (liver and kidney), the following experiments have been made. Normal rabbits, weighing 2,000 to 2,500 grams, were given intravenously 0.2 to 0.5 of a cubic centimeter of pneumococcus broth culture. The experiments have been divided into two series, those dealing with non-perfused organs and those with perfused organs.

In the first series the organs were simply removed from the body after the animal's death. In the second series the animals were killed by a blow on the neck when the clinical symptoms were severe and when pneumococci began to appear in the circulation. Im-

\* Received for publication, January 15, 1914.

<sup>1</sup> Medigreceanu, F., *Jour. Exper. Med.*, 1914, xix, 303.

mediately after the animals were killed they were perfused through the thoracic aorta with 0.8 per cent. saline solution. The perfusion lasted for about half an hour in each case, until the liquid running out became clear and the organs looked pale.

Normal control rabbits were killed in the same way and at the same time as the infected rabbits, or were killed when the infected animals died. All subsequent manipulations of normal and pathological material were carried out in the same manner.

The organs, after removal from the body, were thoroughly ground with half their weight of pure sterilized sand, diluted with their weight of 0.8 per cent. saline solution, and the mixture centrifugalized; the precipitate was washed and again centrifugalized. The joined liquids were finally diluted with 0.8 per cent. saline solution so as to have a concentration in 100 cubic centimeters of about 0.3 to 0.5 of a gram of total nitrogen for the liver and about 0.2 of a gram of total nitrogen for the kidney. Portions were taken for the determination of the total nitrogen, the total filterable nitrogen, and the amino nitrogen. The solutions, covered with a thick layer of toluol in narrow cylinders of the same diameter and thickness, were then submitted to autolysis at 37° C. During the course of autolysis, portions were withdrawn from time to time and the total filterable nitrogen and the amino nitrogen content were estimated. The total nitrogen was estimated according to the Kjeldahl-Gunning method, the filterable nitrogen by precipitating the coagulable proteins by heat and acetic acid, the amino nitrogen by Van Slyke's method.<sup>2</sup>

The filterable nitrogen was estimated to obtain information with regard to the disintegration of the complex protein molecules. The amino nitrogen should afford further knowledge concerning the extent of autolysis of the lower proteinic products, the peptones and the free amino acids. The results are shown in the protocols that follow.

<sup>2</sup> Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 275.

## Experiment I.

Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis. <sup>3</sup>			
Total original nitrogen.	Amino nitrogen.	Total nitrogen.	Amino nitrogen.	Total nitrogen.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Amino nitrogen.
0.3440	0.0888	25.8	0.0348	39.2	0.1370	39.8	0.0782
0.3600	0.0938	26.0	0.0376	40.0	0.1790	49.7	0.1132

Experiment II.							
Liver from normal rabbit				Liver from pneumococcus rabbit			
0.4800	0.1800	37.5	0.0537	29.7	0.2330	48.5	0.1008
0.416	0.1400	33.6	0.0514	36.7	0.2560	61.5	0.1521

## Experiment II.

43.2	0.3300	68.7	0.210	63.6	Death of infected animal 33½ hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed at same time.
59.4	0.2859	68.0	0.1714	60.0	16 hrs elapsed between death of animals and beginning of autolysis.

<sup>3</sup> Autolysis at 37° C. lasted 19 hours in experiment I, and 20 hours in experiment II.

<sup>4</sup> Autolysis at 37° C. lasted 62 hours in experiment I, and 160 hours in experiment II.

*Experiment III.*Filterable nitrogen before autolysis.<sup>5</sup> Filterable nitrogen after autolysis.<sup>6</sup>

Total original nitro- gen, gm. in 100 c.c.	Amino nitrogen.	Total nitrogen.	Amino nitrogen.	Total nitrogen.		Amino nitrogen.		Remarks.
				Per cent. of Gm. in total fil- terable 100 c.c. nitro- gen.				
0.4000	0.1160	29.0	0.0431	37.1	0.2224	55.6	0.1395	Liver from normal rabbit
0.2908	0.0936	32.2	0.0326	34.8	0.1760	60.5	0.1080	Liver from pneumococcus rabbit

*Experiment IV.*

0.3930	0.1210	30.8	0.0413	Liver from normal rabbit	54.6	0.3390	86.2	0.2028	59.9
0.3600	0.0868	24.1	0.0311	Liver from pneumococcus rabbit	51.1	0.2000	55.5	0.1196	59.8

<sup>5</sup> Autolysis at 37° C. lasted 33 hours in experiment III, and 23 hours in experiment IV.

<sup>6</sup> Infected rabbit died 40 hrs. after inoculation. Pneumococci present in blood. Normal rabbit was killed 2 hrs. later.

13 and 15 hrs. respectively elapsed between death of animals and beginning of autolysis.

## Kidney.

## Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis. <sup>7</sup>				Remarks.	
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.			
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.		
0.2320	0.0890	38.3	Kidneys from 2 normal rabbits	0.0255	28.1	0.1550	66.8	0.0695	44.8	Infected rabbits died 27 and 33½ hrs. respectively after inoculation. Pneumococci present in blood. Normal rabbits killed when infected animals died.
0.2550	0.1130	44.3	Kidneys from 2 pneumococcus rabbits	0.0315	27.7	0.1640	64.3	0.1111	67.7	8 and 16 hrs. respectively elapsed between death of animals and beginning of autolysis.

## Experiment II.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis. <sup>7</sup>				Remarks.	
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.			
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.		
0.2180	0.0704	32.3	Kidneys from 2 normal rabbits	0.0228	32.4	0.1370	62.8	0.0934	68.1	Infected rabbits died 18 and 19 hrs. respectively after inoculation. Pneumococci present in blood. Normal rabbits killed when infected animals died.
0.2590	0.0803	31.0	Kidneys from 2 pneumococcus rabbits	0.024	27.8	0.1530	59.1	0.1104	72.1	13 and 14 hrs. respectively elapsed between death of animals and beginning of autolysis.

<sup>7</sup> Autolysis at 37° C. lasted for 40 hours in both experiments.

## SERIES B.

*Perfused Organs.  
Liver.  
Experiment I.*

Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis. <sup>a</sup>				Amino nitrogen.		Filterable nitrogen after autolysis. <sup>a</sup>				Amino nitrogen.		Remarks.	
Total original nitrogen.	Amino nitrogen.	Total nitrogen.	Amino nitrogen.	Total nitrogen.	Per cent. of Gm. in total filterable 100 c.c. nitrogen.	Gm. in original 100 c.c. nitrogen.	Per cent. of Gm. in total filterable 100 c.c. nitrogen.	Total nitrogen.	Per cent. of Gm. in total filterable 100 c.c. nitrogen.	Gm. in original 100 c.c. nitrogen.	Per cent. of Gm. in total filterable 100 c.c. nitrogen.	Total nitrogen.	Per cent. of Gm. in total filterable 100 c.c. nitrogen.	Gm. in original 100 c.c. nitrogen.	Per cent. of Gm. in total filterable 100 c.c. nitrogen.		
0.2115	0.067	31.7	0.0168	25.0	0.0721	34.0	0.0406	56.3	0.1128	53.3	0.0678	60.1					
0.3660	0.1020	27.8	0.0379	37.9	0.2230	61.0	0.1335	59.8	0.3210	87.8	0.2170	67.7	About 24 hrs. elapsed between death of animals and beginning of autolysis.				
<i>Experiment II.</i>																	
0.2566	0.0910	35.4	0.0249	27.3	0.1124	43.9	0.0579	51.5	0.1463	56.9	0.0898	61.3	Infected rabbit was perfused 23 hrs. after inoculation. Normal rabbit was perfused $\frac{1}{2}$ hr. previously.				
0.3020	0.063	20.8	0.0249	39.5	0.1520	50.3	0.0935	61.5	0.2510	83.1	0.1576	62.8	About 24 hrs. elapsed between death of animals and beginning of autolysis.				

HOSPITAL.

*Experiment III.*

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis. <sup>10</sup>				Filterable nitrogen after autolysis. <sup>11</sup>			
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.	
	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.
0.5126	0.1210	23.6	0.0450	37.1	Liver from normal rabbit	0.3100	60.5	0.1925	57.1	0.3650	71.2	0.2915
0.2844	0.060	21.0	0.0271	45.2	Liver from pneumococcus rabbit	0.1430	50.2	0.0885	61.8	0.2020	71.0	0.1292

<sup>10</sup> Autolysis at 37° C. lasted 19 hours.

<sup>11</sup> Autolysis at 37° C. lasted 62 hours.

About 4 hrs. elapsed between death of animals and beginning of autolysis.

## Kidney.

## Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.			Filterable nitrogen after autolysis. <sup>12</sup>			Remarks.		
	Total nitrogen.		Amino nitrogen.	Total nitrogen.		Amino nitrogen.			
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.			
0.2120	0.080	37.7	Kidneys from 2 normal rabbits 0.0303	37.7	0.1530	72.1	0.0937	61.2	Infected rabbits were perfused 23 and hrs. respectively after inoculation. Normal rabbits were perfused 1 hr. previously.
0.1916	0.0726	37.9	Kidneys from 2 pneumococcus rabbits 0.0260	35.8	0.1327	69.2	0.0792	59.7	About 24 hrs. elapsing between death of animals and beginning of autolysis.

## Experiment II.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.			Filterable nitrogen after autolysis. <sup>12</sup>			Remarks.		
	Total nitrogen.		Amino nitrogen.	Total nitrogen.		Amino nitrogen.			
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.			
0.2687	0.0978	36.3	Kidneys from 2 normal rabbits 0.0334	34.2	0.1920	71.4	0.1480	77.1	Infected rabbits were perfused 24 and hrs. respectively after inoculation. Normal rabbits were perfused 1 hr. previously.
0.1724	0.060	34.8	Kidneys from 2 pneumococcus rabbits 0.0189	31.5	0.1310	76.1	0.0930	71.2	About 7 hrs. elapsing between death of animals and beginning of autolysis.

<sup>12</sup> Autolysis lasted for 40 hours in both experiments.

## SUMMARY AND CONCLUSIONS.

*Liver.*—The amount of filterable nitrogen in the samples before autolysis is too variable, both in the normal livers and in the livers from pneumococcus infections in the rabbit, for a constant difference to be obtained.

The amino nitrogen before autolysis is generally slightly increased in the livers of the infected animals, especially in the perfused specimens.

Most of the experiments show an increased disintegration of the livers of the infected animals during the first stages of proteolysis (twenty to thirty hours), both in the non-perfused and the perfused specimens. This increased disintegration is generally more evident for the complex proteins (filterable nitrogen) than for the lower products (amino nitrogen).

In the later stages the proteolysis of the blood-containing livers of the normal and the infected rabbits tends to an equalization, or the relations reverse, the proteolysis of the normal samples becoming more advanced. In the perfused specimens the differences in the rate of proteolysis are the same as in the earlier stages.

*Kidney.*—The rate of proteolysis shows no constant differences in the normal and infected tissues.

WILLIAMSON

AMERICAN BENT BRIGHAM  
HOSPITAL.

## THE EFFECTS OF EARLY EXTIRPATION OF THE THYMUS IN ALBINO RATS.\*

By ALWIN M. PAPPENHEIMER, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons,  
Columbia University, New York.)

### INTRODUCTION.

Marked differences have been observed in the character and intensity of the changes that follow extirpation of the thymus. These differences may be attributed in part to variations in the conduct of the experiments, but in part they appear to depend upon the particular species of animal used. Most of the recent experimental work has been done upon dogs and rabbits. Fewer, and for the most part poorly controlled observations have been made upon other animals,—goats, guinea pigs, cats, chickens, and frogs,—but they have not led to conclusive results.

The effect of removal of the thymus gland in white rats has not previously been investigated. For this reason, and because it was expected that disturbances in the calcium metabolism resulting from the loss of the gland at an early age might be manifested by changes in the teeth, a systematic study has been made during the past year of the effects of thymectomy upon white rats. The experiments were interrupted last spring by the outbreak of an epidemic, due to infection by a bacillus of the enteritis group, which destroyed many of the rats.<sup>1</sup> The work has been hampered by the sporadic occurrence of this infection, and to a lesser degree by the chronic lung disease which is so often found in rats. As the result of a year's work, therefore, we have only a limited number of uninfected rats from which conclusions as to growth and nutrition may be drawn. However, the experiments have been carefully controlled, and we feel justified in reporting the data, especially since the known nor-

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<sup>1</sup> A study of the bacteriological and pathological findings in this disease will appear in a forthcoming number of the *Journal of Infectious Diseases*.

mal variations in growth and weight, as recently worked out in detail by Hatai (1) and Jackson (2), serve as further controls. These figures emphasize the need for caution in drawing inferences from slight differences in weight. This is true even for animals of the same litter, maintained under apparently identical conditions.

The literature relating to the experimental extirpation of the thymus has been collected in the exhaustive reviews of Hamer (3), Wiesel (4), Klose (5), Biedl (6), Matti (7), and Hart (8). Since the effect of removing the thymus of rats has not previously been studied, reference will be made only to the observations which bear upon our work.

#### METHODS AND TECHNIQUE.

The removal of the thymus was performed upon young rats ranging in weight from ten to twenty-five grams. When possible the age was accurately determined, but in some litters the age had to be estimated. Probably none of the operated animals were over twenty-one days old when the gland was removed. Two or more rats from each litter were reserved as controls, and kept in the same cage as the operated rats.

The operation was performed aseptically under ether anesthesia. A median incision was made in the lower part of the neck and continued over the upper half of the thorax. The sternum was split as nearly as possible in the median line as far as the fourth or fifth intercostal space, and the edges were retracted. The triangularis sterni muscle was separated, exposing the thymus, which was carefully dislodged from below upwards with fine blunt forceps, removed when possible, without laceration or separation of the lobes. The friability of the gland made this the most difficult part of the operation. The sternum and skin were sutured with silk. Healing in almost all cases took place rapidly and without suppuration. It was found advantageous to use a simple positive pressure apparatus, to prevent collapse of the lung when the pleura was accidentally injured, and to institute artificial respiration when necessary.

In the earlier experiments the control rats were also subjected to etherization and the sternum was incised without removal of the

thymus. It was found later that the operation had practically no immediate effects, and this precaution was therefore considered unnecessary.

After the end of the nursing period; the rats were fed principally on bread, sunflower seed, and greens. Meat was not given, and the feeding of milk was given up, since it seemed to favor the spread of infection.

The operated rats together with the controls were kept under observation for varying periods up to six months, and were then killed. They were weighed weekly.

At autopsy the organs of the neck and thorax were removed together. After fixation the lungs and lower half of the heart were dissected off, and the rest of the tissue was embedded in paraffin and cut in series of 10 micra in thickness. In the earlier litters each section was mounted, but later only every tenth section was examined. When there was doubt as to the nature of the tissue, the intermediate sections were also studied.

The other organs were fixed in Zenker's fluid, and stained with hematoxylin and eosin. The bones and teeth were fixed in formalin and decalcified in phloroglucin nitric acid, or in some cases, in Müller's fluid. The adrenals were fixed in Müller-formol solution.

#### EXPERIMENTAL PART.

26 litters, comprising 118 rats, were used for this study. Of these, 82 were thymectomized, the remainder serving as controls. 19 rats died during or soon after the operation. 5 litters, including 14 operated rats and 11 controls, are still under observation. In 18 of the remaining 49 rats, extirpation was proved to have been complete by serial section through the entire neck organs. In one of the rats accessory thymus tissue was found. 5 of the 18 rats showed lesions characteristic of infection with the bacillus of the enteritis group mentioned above, and are excluded on this account.

The following abbreviated protocols, therefore, refer only to rats in which extirpation was proved to have been complete, in which no accessory tissue was present, and which were shown by microscopic study to be free from infection.

The data given in table I afford a decisive answer to the question

TABLE I.

## Summary of Protocols.

Litter.	Sex.	Age at operation.	Weight at operation.	Weight at death.	Duration of life after operation.	Remarks.
A <sub>2</sub>	F.	14 dys.	14.1 gm.	58.5 gm.	31 dys.	Killed with chloroform
A <sub>3</sub>	M.	14 dys.	16.3 gm.	66.0 gm.	31 dys.	Killed with chloroform
A <sub>5</sub>	F.	14 dys.	14.6 gm.	62.0 gm.	31 dys.	Control: sternum incise
A <sub>6</sub>	F.	14 dys.	14.6 gm.	65.0 gm.	31 dys.	Control.
B <sub>2</sub>	M.	15 dys.	16.1 gm.	86.9 gm.	43 dys.	Killed with chloroform
B <sub>6</sub>	M.	15 dys.	16.4 gm.	98.6 gm.	43 dys.	Control: sternum incise
E <sub>2</sub>	M.	10 dys.	12.0 gm.	77.5 gm.	58 dys.	Killed with chloroform
E <sub>6</sub>	F.	10 dys.	12.0 gm.	59.0 gm.	58 dys.	Control.
F <sub>1</sub>	F.	10 dys. (?)	18.5 gm.	70.0 gm.	99 dys.	Killed with chloroform
F <sub>4</sub>	F.	10 dys.	18.0 gm.	111.0 gm.	99 dys.	Control (infected).
M <sub>3</sub>	F.	13 dys.	11.0 gm.	33.0 gm.	70 dys.	Spleen removed 11 days after thymectomy.
M <sub>7</sub>	F.	13 dys.	11.0 gm.	49.0 gm.	82 dys.	Control: spleen removed same time as M <sub>3</sub> .
O <sub>1</sub>	M.	20 dys.	21.0 gm.	107.0 gm.	120 dys.	Killed with chloroform
O <sub>2</sub>	M.	20 dys.	21.5 gm.	106.0 gm.	120 dys.	Killed with chloroform
O <sub>3</sub>	F.	20 dys.	23.0 gm.	87.0 gm.	131 dys.	Killed with chloroform
O <sub>5</sub>	M.	20 dys.	23.5 gm.	122.5 gm.	120 dys.	Control.
O <sub>6</sub>	F.	20 dys.	21.5 gm.	83.0 gm.	131 dys.	Control.
P <sub>1</sub>	F.	23 dys.	24.5 gm.	137.0 gm.	130 dys.	Killed with chloroform
P <sub>2</sub>	F.	23 dys.	24.5 gm.	111.0 gm.	130 dys.	Killed with chloroform
P <sub>3</sub>	F.	23 dys.	24.5 gm.	118.0 gm.	130 dys.	Control.
S <sub>2</sub>	F.	14 dys.	17.0 gm.	63.0 gm.	95 dys.	Minute thymus rest (?)
S <sub>6</sub>	M.	14 dys.	18.0 gm.	72.0 gm.	90 dys.	Control.

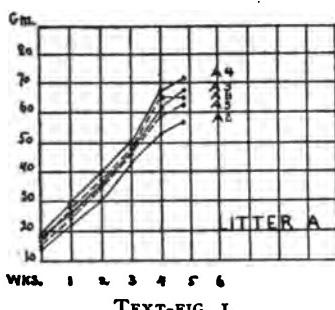
whether the thymus in rats is a vital organ. The period of survival after operation ranged from 45 to 131 days. The animals of the last group (litters O and P) showed no evidence of illness at the time. Among the litters at present under observation are two which have survived the operation for 170 days and are still in good health.

It is therefore proved that whatever influence the thymus may have upon growth and nutrition in the rat, it is not essential to life. Sexual maturity, according to Slonaker, is attained at ten weeks, although the maximum weight is not reached until after the first year. The period of observation is therefore sufficiently prolonged in the case of litters E, M, F, S, O, and P, to carry the animals well past the onset of puberty, at which time in rats (Jackson) and

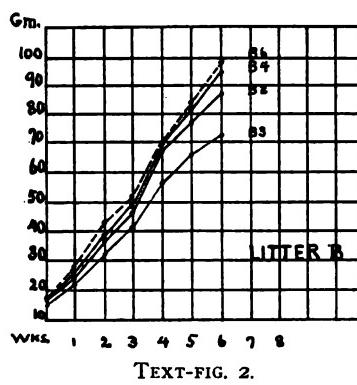
abbits (Söderlund and Backman (9)), the thymus has begun its normal involution, and is probably no longer at the height of its functional activity. It is not reasonable to suppose that the fatal effects of thymus deprivation would appear only after the anatomic regression of the organ was well under way.

#### INFLUENCE OF THYMECTOMY UPON GROWTH AND NUTRITION.

An analysis of the weight curves (text-figures 1 to 8)<sup>2</sup> does not indicate a definite influence of thymectomy upon growth.

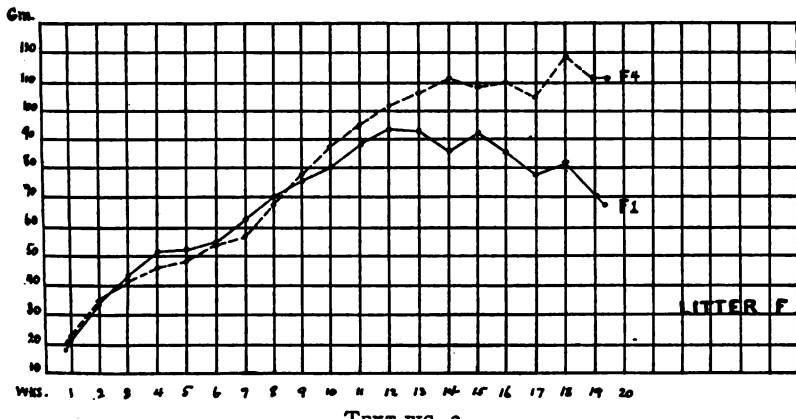


TEXT-FIG. 1.



TEXT-FIG. 2.

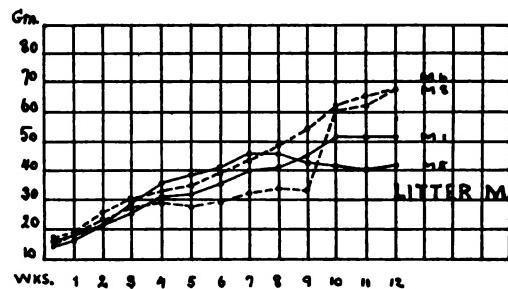
The ten completely thymectomized rats in which microscopic examination showed no evidence of infection were A<sub>2</sub>, A<sub>3</sub>, B<sub>2</sub>, E<sub>2</sub>, F<sub>1</sub>, O<sub>1</sub>, O<sub>2</sub>, O<sub>3</sub>, P<sub>1</sub>, and P<sub>2</sub>. Two of the rats, A<sub>2</sub> and B<sub>2</sub>, showed



TEXT-FIG. 3.

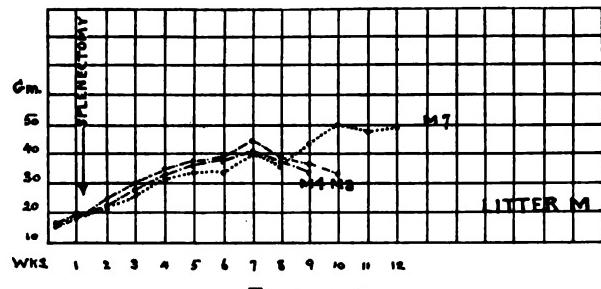
<sup>2</sup>In the charts the unbroken lines represent operated animals, the broken lines controls.

a slight retardation of growth as compared with healthy controls of the same litter. The differences in weight at the time when



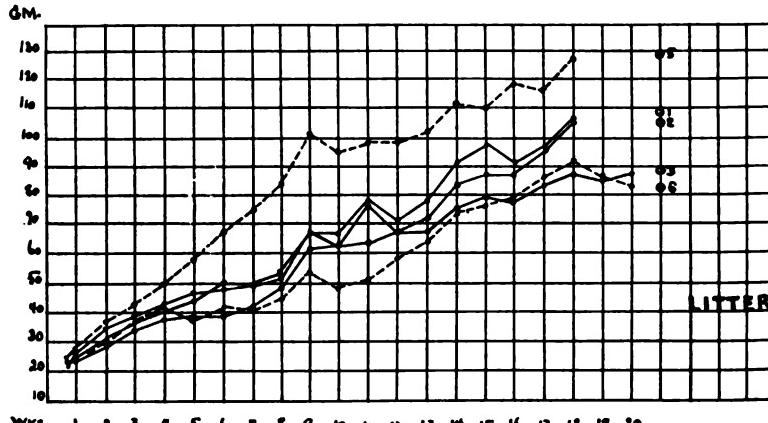
TEXT-FIG. 4.

animals were killed were insignificant and fall easily within the range of normal litter variation as determined by Jackson.



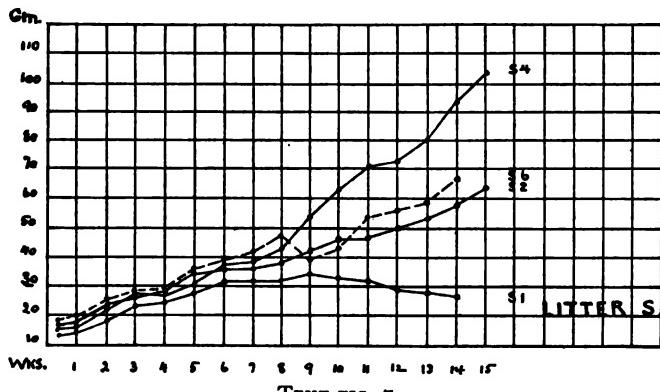
TEXT-FIG. 5.

A<sub>3</sub> exceeded both controls. Rats O<sub>1</sub>, O<sub>2</sub>, and O<sub>3</sub> were mi-



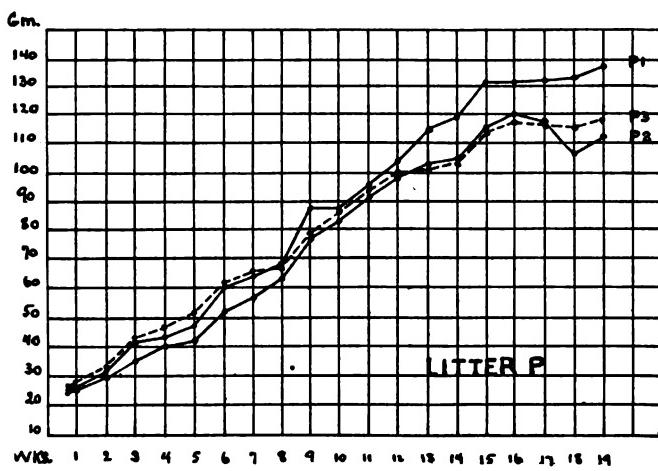
TEXT-FIG. 6.

between the two controls in weight. Rat P<sub>1</sub> was above, Rat P<sub>2</sub> below the control. Rat F<sub>1</sub>, ninety-nine days after thymectomy,



TEXT-FIG. 7.

weighed forty grams less than the normal control, F<sub>4</sub>, but even extreme variations such as this are apparently not uncommon in rats raised under laboratory conditions.<sup>3</sup>



TEXT-FIG. 8.

On the basis of these facts, and taking into consideration our data relating to rats in which small lobules of thymic tissue were

<sup>3</sup> Jackson, C. M., *Am. Jour. Anat.*, 1913, xv, 1. Compare table I, litter M<sub>7</sub>, in which the weights ranged at 44 days from 23.5 to 71.8 gm. The variations may of course be due to unrecognized pathological conditions.

found, but in which there must have been a marked functional deficiency, one must conclude that removal of the thymus in rats does not noticeably affect growth and nutrition.

#### BEHAVIOR OF THE OPERATED RATS.

If we leave out of consideration all symptoms that could be attributed to accidental infection and that could be duplicated in normal rats experimentally infected with *Bacillus enteritidis*, no abnormalities in behavior were noted. Particularly to be emphasized is the absence of nervous irritability, tremors, convulsions, and stupor. There was not the slightest evidence of mental deterioration in any of the operated animals, nor was there anything peculiar in their appearance or posture.

#### LOCAL CHANGES.

The large space left by the removal of the gland becomes rapidly filled by adipose tissue, which for a long time, preserves the characteristic embryonic fat lobules.

The regional lymphatic glands in the operated rats show a marked hyperplasia. This is accompanied frequently by a distension of the lymph sinuses, with exfoliation of endothelial cells. In some cases the sinuses were found filled with red blood cells, many enclosed in phagocytes.

As regards the behavior of small fragments of thymus tissue removed at operation, only a few general statements can be made, as a study of the minute regenerative changes do not fall within the scope of the work. The general capacity of small fragments of thymic tissue to regenerate did not appear to be so marked as the statements made by Klose and others led me to believe. The minute rests found in serial sections were often extremely minute and showed no evidence of active proliferation. They were composed of densely packed small cells, among which were a few composed of larger, paler epithelial cells. Fully formed, concentrically arranged Hassall bodies were only exceptionally seen. The identification of small masses of tissue as thymus was therefore somewhat difficult, and rested upon the absence of peripheral lymph sinuses and the presence of the epithelial cell complexes. Because of the closer crowding of the cells, the staining of the thymus tissue was somewhat deeper than that of the adjacent lymph nodes.

A further differential point observed was the absence of necrotic lesions in the thymic tissue in rats infected with *Bacillus enteritidis*. The surrounding lymphoid tissue in these rats regularly contained areas of focal necrosis, but in not a single instance was the thymus or the rest of thymic tissue similarly affected. This is in accord with the well known insusceptibility of the thymus to tuberculosis and other specific infections.

#### THE INFLUENCE OF THYMECTOMY UPON OTHER ORGANS.

*Skeletal System.*—Particular attention was given to possible alterations in the bones and teeth. The femur, tibia, upper and lower incisors, and in some cases, the ribs, were examined. The methods used included partial decalcification in Müller's fluid, and staining with hematoxylin and carmine after Schmorl.

I have failed to observe any characteristic qualitative changes which might be brought into relation with the operation. Whenever marked retardation of growth occurred, the bones were shorter, the epiphyseal cartilage narrower, the cortical and trabeculae more slender, and the marrow spaces larger than in the controls. In not a single instance, however, was there marked gross deformity, nor was there any irregularity in the processes of ossification. Rachitis-like lesions like those described by Klose (10) and Matti (11) in dogs, and by Soli (12) in rabbits were wanting.

The teeth also showed no irregularities in the calcification of the dentine, and none of the animals that were observed over a long period showed spontaneous infarctions such as have been described by Erdheim (13) and Toyofuku (14) in parathyroidectomized rats.

In the absence of chemical studies it seems unwarranted to deny the influence of the thymus in rats upon skeletal and dental calcification. The foregoing observations, however, certainly afford no histological evidence pointing to such an influence.

*Adrenals.*—There has been brought forward considerable pathological and experimental evidence indicating a correlation or antagonism between the thymus and the adrenals. Matti (11) states that in thymectomized dogs, there was regularly observed, on macroscopic examination, a hypertrophy of the adrenal medulla. Histologically, there were found the changes described by Stoerk

and von Haberer (15), as characteristic of hypertrophic medullary tissue. Other references to the condition of the adrenals after thymectomy are less definite. Soli found the adrenals diminished in weight in six chickens and increased in five; in eight guinea-pigs it was increased in six and diminished in two. Nordmann (16) describes a hyperemia. Lucien and Parisot (17) found a slight diminution in the absolute weight, and no appreciable change in the relative weight of the adrenals after thymectomy.

Hyperplasia of the thymus after adrenalectomy is described by Calogero (18), Bonnet (19), and Auld (20), and, as pointed out by Hedinger (21), Hart (22), Wiesel (23), Pappenheimer (24), and others, occurs frequently in Addison's disease.

I have not found definite alterations in the adrenals in thymectomized rats, either in weight or in histological structure. The chromaffin staining tissue was invariably abundant, but not more so than in the controls. Changes in the chromaffin cells which could be interpreted as indicating a hypertrophy were not found. The cortex also showed no structural difference from that of the control.

TABLE II.  
*Weight of Adrenal Glands.*

Rat.	Sex.	Operation.	Absolute weight.	Relative weight
M <sub>1</sub>	F.	Thymectomy (I) <sup>4</sup>	0.030 gm.	I-173
M <sub>5</sub>	F.	Thymectomy (C) <sup>4</sup>	0.020 gm.	I-210
M <sub>6</sub>	M.	Control	0.020 gm.	I-333
M <sub>7</sub>	F.	Splenectomy	0.020 gm.	I-245
M <sub>8</sub>	M.	Control	0.011 gm.	I-690
O <sub>1</sub>	M.	Thymectomy (C)	0.022 gm.	I-48
O <sub>2</sub>	M.	Thymectomy (C)	0.024 gm.	I-43
O <sub>3</sub>	F.	Thymectomy (C)	0.017 gm.	I-51
O <sub>4</sub>	M.	Control	0.024 gm.	I-50
O <sub>5</sub>	F.	Control	0.021 gm.	I-39
P <sub>1</sub>	F.	Thymectomy (C)	0.030 gm.	I-45
P <sub>2</sub>	F.	Thymectomy (C)	0.025 gm.	I-44
P <sub>3</sub>	F.	Control	0.028 gm.	I-42
S <sub>1</sub>	F.	Thymectomy (C)	0.013 gm.	I-20
S <sub>2</sub>	F.	Thymectomy (C?)	0.021 gm.	I-30
S <sub>4</sub>	M.	Thymectomy (I)	0.027 gm.	I-32
S <sub>5</sub>	F.	Splenectomy	0.015 gm.	I-32
S <sub>6</sub>	M.	Control	0.020 gm.	I-36
U <sub>4</sub>	M.	Thymectomy (I)	0.020 gm.	I-18
U <sub>7</sub>	M.	Control	0.017 gm.	I-22

<sup>4</sup> In the tables, I = incomplete, and C = complete.

renals, although special staining of the lipoids was not attempted. The absolute and relative weights of the adrenals in some of the operated litters are appended.

Although at first sight the figures appear to indicate a relative increase in the weight of adrenals in the thymectomized rats, this is probably due to the relatively small weight of many of the operated animals rather than to an hypertrophy. That it cannot be directly attributed to the loss of thymus is indicated by the fact that the weight is relatively high also in the splenectomized rats, M<sub>7</sub> and S<sub>5</sub>. The highest relative weights, moreover, are found in rats M<sub>1</sub> and U<sub>4</sub>, which were infected and cachectic, and in which the extirpation of the thymus was incomplete.

*Testes.*—The condition of the testes in the operated animals and in the corresponding controls is given in table III.

TABLE III.

*Testes.*

Rat.	Age.	Operation.	Weight of testicles.	Size of testicles.	Spermatogenesis.
A <sub>3</sub>	45 dys.	Thymectomy		4.5 mm.	No spermatogenesis.
A <sub>4</sub>	45 dys.	Thymectomy (I)		12.0 mm.	No spermatogenesis.
A <sub>6</sub>	45 dys.	Control		6.5 mm.	No spermatogenesis, More mitotic figures than A <sub>3</sub> and A <sub>4</sub> .
B <sub>2</sub>	58 dys.	Thymectomy (C)	1.51 gm.		Spermatogenesis.
B <sub>4</sub>	58 dys.	Thymectomy (I)	1.45 gm.		Spermatogenesis.
B <sub>6</sub>	58 dys.	Control	1.50 gm.		Spermatogenesis.
E <sub>2</sub>	68 dys.	Thymectomy (C)		16.0 mm.	Active spermatogenesis.
E <sub>3</sub>	68 dys.	Thymectomy (I)		11.0 mm.	No spermatogenesis.
O <sub>1</sub>	140 dys.	Thymectomy (C)	1.456 gm.		Active spermatogenesis.
O <sub>2</sub>	140 dys.	Thymectomy (C)	1.425 gm.		Active spermatogenesis.
O <sub>5</sub>	140 dys.	Control	1.664 gm.		Active spermatogenesis.
S <sub>4</sub>	104 dys.	Thymectomy (I)	1.130 gm.		Fairly active spermatogenesis.
S <sub>6</sub>	104 dys.	Control	0.605 gm.		Fairly active spermatogenesis.
U <sub>4</sub>	79 dys.	Thymectomy (I)	0.180 gm.		No spermatogenesis.
U <sub>7</sub>	79 dys.	Control	0.296 gm.		No spermatogenesis.

In litter A spermatogenesis was not fully established at forty-five days, when the rats were killed. In litter B, at fifty-eight days, both thymectomized and control rats showed spermatogenesis; and this

was true in all the other testes examined, except in litter E. In the development of the testis was further advanced in E<sub>2</sub> than another male of the same litter, E<sub>3</sub>, in which the removal of the thymus was incomplete. The data are not sufficiently numerous to warrant a final conclusion, but as far as they go, they do not indicate a decided effect of thymectomy upon the testes.

The literature bearing on this question contains contradictory statements. The most extensive experiments made to determine the influence of thymectomy upon the development of the testes are those of Paton (25). In guinea pigs removal of the thymus was followed by increase in the average weight of the testes amounting in one series to 46 per cent. Microscopic examinations were not made.

In chickens, rabbits, and guinea pigs Soli (26) obtained opposite results. The testicles in the thymectomized animals killed at five months, were smaller, weighed less than the controls, and failed to show active spermatogenesis. Lucien and Parisot (27) found a transient retardation in the development of the testes.

Klose and Vogt (10) substantiated in dogs the findings of Paton in that they observed a transient hyperplasia of the testes, which disappeared in the cachectic stage. Matti (11), on the other hand, was unable to determine any relation between thymectomy and the onset of spermatogenesis.

In view of these conflicting statements and our negative findings in rats, it seems premature to attribute to the thymus a direct influence upon the male genital glands. That there exists an inverse relation, however, by which the involution of the thymus is retarded after early castration, may be considered proved, and the numerous experiments of Henderson (27), Goodall (28), Calari (29), Ranzi and Tandler (30), and Soli and Gellin (31) are in agreement on this subject.

*Thyroid.*—In many of the operated animals the thyroid showed slight variation in structure from that of the controls. The acini were smaller, the epithelium higher, and the colloid less abundant and less densely stained than in the controls. These differences, however, were not striking, and marked epithelial hyperplasia, with papillary infolding of the epithelium, such as Matti (11) described

in thymectomized dogs, was not seen. The impression gained was that the alterations in the thyroid were not specific, but merely accompanied the arrested development. They were equally manifest in rats whose development was retarded by infections or other causes.

*Spleen.*—Soli found the weight of the spleen in chickens and rabbits unaffected by thymectomy. Lucien and Parisot also found no changes in the weight, but observed in certain cases an increase in the number and size of the follicles. Perrier (32), Klose and Vogt, and Matti have also observed a follicular hyperplasia, followed by atrophy in the athreptic stage.

Klose (5) concludes from these facts that the spleen "may to a certain extent and after a certain interval, take over the function of the ablated thymus." Late extirpation of the thymus, which ordinarily causes only transitory "rachitis-like disturbances of growth," if combined with splenectomy, leads to death within a few weeks. Matti (7) disputes both the facts and the inferences that Klose draws.

I have repeated this experiment in three rats, M<sub>3</sub>, M<sub>4</sub>, and S<sub>3</sub>. In M<sub>3</sub> and M<sub>4</sub>, the spleen was extirpated 11 days after thymectomy. The animals gained steadily in weight until the 47th day after thymectomy, when the weight curve began to decline. M<sub>4</sub> was found dead and partially eaten on the 66th day after operation. M<sub>3</sub> became emaciated and weak, and developed diarrhea. It was killed on the 70th day, and extirpation was proved by serial section to have been complete. A control rat, M<sub>7</sub>, in which the spleen alone had been removed, weighed at this time seventeen grams more than M<sub>3</sub>, but also showed a retardation of growth as compared with the unoperated controls, M<sub>6</sub> and M<sub>8</sub>.

This experiment proves that a rat may live for at least fifty-nine days after extirpation of both thymus and spleen. Since the microscopic examination showed ulcerative lesions in the intestine and inflammatory foci in the lungs, it is probable that death was due to intercurrent infection rather than to the lack of these organs.

In rat S<sub>3</sub>, which was splenectomized fifteen days after removal of the thymus, there was a normal gain in weight surpassing that of a control rat of the same litter (S<sub>5</sub>) in which the spleen alone had

been extirpated, but lagging somewhat behind the two unoperated controls. It was found dead and partially eaten eighty-eight days after thymectomy. Since the neck organs could not be examined serially, it was not possible to be sure that the thymus had completely removed.

The absolute and relative weights of the spleen in our series of animals are given in table IV. There is wide variation in the weights.

TABLE IV.  
*Weight of Spleens.*

Operated rats.		Controls.	
A <sub>2</sub>	0.25 gm.	B <sub>6</sub>	0.325 gm.
B <sub>2</sub>	0.62 gm.		
B <sub>3</sub>	0.22 gm.		
M <sub>1</sub>	0.19 gm.	M <sub>6</sub>	0.205 gm.
M <sub>5</sub>	0.19 gm.	M <sub>8</sub>	0.285 gm.
O <sub>1</sub>	0.397 gm.	O <sub>5</sub>	0.396 gm.
O <sub>2</sub>	0.301 gm.	O <sub>6</sub>	0.238 gm.
O <sub>3</sub>	0.270 gm.		
P <sub>1</sub>	0.357 gm.		
P <sub>2</sub>	0.271 gm.	P <sub>3</sub>	0.292 gm.
S <sub>1</sub>	0.192 gm.		
S <sub>2</sub>	0.425 gm.		
S <sub>3</sub>	0.818 gm.	S <sub>6</sub>	0.560 gm.
U <sub>4</sub>	0.220 gm.	U <sub>7</sub>	0.166 gm.

and those of the thymectomized rats are sometimes heavier and sometimes lighter than those of the controls. The same variation was noted in the histological pictures. The size of the lymphatic follicles in well nourished rats was large; in atrophic animals atrophy of the lymphoid tissue occurred regularly. In no case where the nutrition of operated and control rats was equally good were striking differences found in the histological picture. It seems therefore that thymectomy in rats does not lead to constant or definite alterations in the structure of the spleen.

*Blood.*—The differential blood counts<sup>5</sup> on four operated rats are given in table V. The counts are not sufficiently numerous

<sup>5</sup>I wish to express my gratitude to Miss Gertrude Fisher for making the blood counts. Although the counts were not continued for a longer period than five weeks after the operation, the data are given since they may prove of value to other workers in this field.

TABLE V.

Litter M. Differential Count Made on August 14, 1913, Five and One Half Weeks after Thymectomy.

	Polymor-phonuclears.	Small lym-phocytes.	Large lym-phocytes.	Large mono-nuclears.	Transi-tionals.	Eosino-phils.
M1 Incomplete thymectomy; infection	36	26	22	8	8	0
M2 Complete thymectomy and splenectomy; no infection	46	18	22	10	4	0
M3 Complete thymectomy and splenectomy; no infection	21	38	25	8	8	0
M4 Complete thymectomy.....	28	29	20	11	12	0
M5 Control.....	30	16	43	7	4	0
M6 Control: splenectomy.....	30	27	30	13	5	0
M7 Control.....	35	19	24	17	5	0

Litter S. Operated July 22, 1913.

S1 Complete thymectomy July 24..... July 31..... Aug. 9..... Aug. 14..... Aug. 21.....	17 41 41 34 36	56 16 31 32 26	22 17 16 24 25	12 15 11 3 8	3 11 1 7 5	0 0 0 0 0
S2 Complete(?) thymectomy July 24..... July 31..... Aug. 9..... Aug. 14..... Aug. 21.....	23 39 35 30 31	26 29 18 21 15	30 22 31 27 32	7 5 13 14 14	4 5 3 8 8	0 0 0 0 0
S3 Thymectomy, splenectomy (Aug. 6) July 24..... July 31..... Aug. 9..... Aug. 14..... Aug. 21.....	17 42 33 46 43	45 20 27 34 8	24 26 28 9 17	9 8 4 6 18	4 4 8 5 14	0 0 0 0 0
S4 Control: splenectomy July 24..... July 31..... Aug. 9..... Aug. 14..... Aug. 21.....	13 11 15 44 22	60 76 61 18 46	20 8 17 22 21	4 2 4 11 7	3 3 3 5 4	0 0 0 0 0

TABLE V.—Concluded.

		Polymor-phonuclears.	Small lymphocytes.	Large lymphocytes.	Large mononuclears.	Transi-tionals.	Eosin.
S5	Control: no infection						
	July 24.....	14	68	13	1	4	
	July 31.....	8	58	30	1	3	
	Aug. 9.....	16	53	18	6	2	
	Aug. 14.....	15	46	20	12	7	
	Aug. 21.....	25	25	35	7	8	

Litter W.<sup>6</sup> Operated November 22, 1913. Still under Observation.

W1	Thymectomy						
	Oct. 27.....	46	14	14	26 <sup>7</sup>		
	Nov. 6.....	28	39	22	11		
	Nov. 12.....	42	24	26	8		
	Nov. 17.....	40	32	20	8		
	Nov. 25.....	50	25	29	6		
W2	Thymectomy						
	Oct. 27.....	33	48	10	9		
	Nov. 9.....	35	54	3	8		
	Nov. 12.....	43	27	31	9		
	Nov. 17.....	48	14	27	9		
	Nov. 25.....	32	15	46	7		
W3	Thymectomy						
	Oct. 27.....	60	18	14	8		
	Nov. 1.....	58	20	16	6		
	Nov. 12.....	43	21	31	5		
	Nov. 17.....	49	15	31	5		
	Nov. 25.....	49	10	34	8		
W4	Control .....						
		22	62	9	7		
		13	46	36	5		
		28	35	14	14		
		37	20	29	14		
		44	32	18	6		
W5	Control .....						
		13	52	26	9		
		11	63	19	7		
		44	25	24	7		
		23	40	33	4		
		40	25	27	4		

warrant a minute analysis or to permit of generalizations. There is an evident tendency, particularly in litter S, towards the occurrence of a relatively high proportion of polymorphonuclears during the first weeks after operation. Since the wounds healed aseptically

<sup>6</sup> The total leucocyte count in this litter showed no differences between operated rats and controls. Leucocyte counts were also made on the rats of litters S and P, and no variation from the normal was found.

<sup>7</sup> Large mononuclears and transitionals were counted together.

and the polynucleosis persisted after complete healing, the change in the blood picture is probably due to the loss of the thymus rather than to the effect of the operation.

Previous studies of the blood changes in other animals following thymectomy are contradictory. Seiler (33), who made blood counts upon one of Matti's operated litters of dogs, found a delay in the establishment of the normal percentage of polynuclears; the lymphocytic count remained high for a longer period than in the controls. Matti attributes this to the general retardation in development. However, an inspection of the actual counts made by Seiler several months after the operation shows that the percentage of polymorphonuclears in the thymectomized dogs was slightly higher (72 to 81 per cent.) than in the two controls (65 to 68 per cent.).

Lampé and Liesegang (34), who carried out blood studies on dog's animals, found a progressive diminution of the lymphocytes to far below their normal values. This is more in accord with my findings, and is also in harmony with the well known occurrence of relative lymphocytosis in Status lymphaticus, Basedow's disease, and other alterations of the ductless glands commonly associated with hyperplasia of the thymus.

#### CONCLUSIONS.

It has been shown that white rats may survive complete extirpation of the thymus for at least 131 days, even when the operation is performed within the first two weeks of life.

Removal of the thymus does not produce an arrest or retardation of body growth and development.

Qualitative changes in the skeletal system or teeth have not been found. In emaciated, weak animals osteogenesis is less active than in healthy rats, and the long bones are smaller and more delicate in structure. Such quantitative differences appear to depend upon the general nutrition, are equally pronounced in rats whose development is retarded from other causes, and cannot be referred specifically to loss of thymus function.

No constant or characteristic alterations were detected in the spleen, testes, adrenals, or thyroid. Whatever functional correla-

tions may exist between thymus and any or all of these organs, not evident from the occurrence of histological changes after removal of the thymus.

The relative proportion of lymphocytes in the blood is altered for the first few weeks after the operation. We have determined how long this alteration in the leucocytic formula per-

Since this paper went to press an article has appeared by Klose<sup>1</sup> describing briefly the results of thymus extirpation in pigs, rats, and chickens. In rats thymectomized on the fourteenth day there followed a progressive cachexia terminating in death eight to ten weeks. Disturbances in ossification, which macroscopically and microscopically were identical with those of rickets, developed also in the ribs and long bones. Some of Klose's litters failed to show these lesions, and this negative result is explained as having been due to the presence of thymic tissue in the thyroid gland.

The observations of Klose are in direct contradiction to the negative results described in this paper. Since the possibility of accessory thymus tissue either within the thyroid or elsewhere was carefully excluded in my experiments, the discrepancy between my findings and those of Klose cannot be explained on this basis.

Since this paper was sent to the publisher I have studied additional rats which were killed 185 days after operation. My examination of a complete series of the neck organs, including the thyroid, failed to show any tissue which could be interpreted as thymus. The bones showed no rachitic changes.

The infective origin of rachitic and osteomalacic lesions in children has been established by Morpurgo. Although reference is made by Klose to Morpurgo's work, the data given by him do not allow one to judge whether this infection was definitely excluded.

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<sup>1</sup> Klose H., Thymusdrüse und Rachitis, *Centralbl. f. allg. Path.*, 1914.

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## THE RELATION OF THE GASTRO-INTESTINAL TRACT AND CONTENTS TO THE BODY-WEIGHT IN RABBITS.\*

By A. E. LIVINGSTON.

(*From the Physiological Laboratory of the Medical College of Cornell University, Ithaca.*)

In some experimental work on rabbits in this laboratory, in which the results were based on the body-weight, it has been found necessary to avoid the error introduced by the variation in the weight of intestinal, stomach, and bladder contents. In view of a possible wider interest in the data collected for our use, we report them in this paper.

Joseph<sup>1</sup> examined one hundred rabbits for the purpose of determining the proportion of body-weight which should be allowed for stomach and cecum contents when the dosage of various drugs is based on the weight of the animal. He gives figures for stomach and cecum contents of males and females, and for the stomach and cecum minus contents. The results given in this paper deal collectively with the contents of the stomach, small intestine, cecum, large intestine, and rectum, of both sexes; the figures also include the weights of the organs after the contents had been removed, in normal, castrated, and thyroidectomized animals. The data in the literature upon this subject are not sufficiently complete to meet all conditions nor to justify the formation of a rule which will cover all cases; hence it became necessary to determine the exact weights in each animal used. The error in body weight due to bladder contents may be obviated by applying digital pressure on the abdomen in the region of the bladder as soon as the animal is dead and the muscles are relaxed. This method was used in every case, and throughout the paper the weight after removal of urine is spoken of as gross body-weight. The problem of eliminating the error

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<sup>1</sup> Joseph, D. R., *Jour. Exper. Med.*, 1909, xi, 36.

introduced by inclusion of the stomach and intestinal contents made more difficult. The animal was weighed before and after the stool was expelled, and then the whole tract from the cardiac end of the stomach to the lower end of the rectum was removed, with as much of the mesentery as possible. The tract was then weighed with its contents. The removal of the latter was accomplished by first cutting the tract at the junction of its several parts; then the stomach was opened along its greater curvature and the material within scraped away without the removal of any of the mucous membrane. The remaining parts were placed on a smooth board and by means of a small piece of sheet cork, which does not cut the tissue, the contents were forced out by pressure along the outside. This continued until all the material was removed. The stomach and intestines, now minus their contents, were weighed collectively and their weight was subtracted from that obtained before the removal of the contents, thus giving the content weight which when deducted from the gross body-weight gave what we shall call the reduced body-weight. The method of clearing the intestines of their contents by allowing a current of water to pass through introduced a considerable error by the water that remains in the folds of the lining mucous membrane, while with the present method a comparatively small error is made. The animals were killed by coagulation when it happened to be most convenient, without respect to feeding time, and this undoubtedly accounts to some extent for the variation in the weight of gastro-intestinal contents.

Among the animals examined there were thirty-one castrated males, thirty-three normal males, twenty-one spayed and thirty-one normal females, five thyroidectomized males, and six thyroidectomized females. In the tables the averages are arranged in ascending order with respect to the reduced body-weight. In column 1 of the four tables the numbers indicate the limits between which the reduced body-weight varies.

In tables I to IV it will be observed that the absolute weights decrease directly with the reduced body-weights, while the weights per cent, as seen in columns 8 and 9, vary inversely with the reduced body-weight. In table V the averages of the twenty-one spayed females are compared with those of the thirty-one controls. It will be re-

that the reduced body-weight is slightly more in the spayed than in the normal animals and that the absolute weights vary even with this slight difference. In table VI where the averages of the thirty-three normal and thirty-one castrated males are compared, the absolute weights vary with the reduced body-weight without exception. In table VII where the averages of the whole group of sixty-four castrated and control males are compared with those of the whole group of fifty-two spayed and control females, it will be noted that the average of the gastro-intestinal tract plus the contents is slightly more in the males that have a lower average for reduced body-weight. This is due to the contents, as shown by column 6. The weight of the tract alone varies with the reduced body-weight.

In table VIII is given a comparison of the averages of the sixty-four normal animals, both males and females, with fifty-two operated males and females, and here again there is no exception to the rule that the variation in reduced body-weight, although small, is accompanied by a direct variation of the gross body-weight, weight of gastro-intestinal tract plus its contents, the same tract minus its contents, and the contents alone.

The same is true without exception in table IX, where the thyroid-ectomized males and females are compared.

In tables V to IX there is a constant apparent contradiction to tables I, II, III, and IV, in that the weights per kilo as shown in columns 8 and 9 vary directly with the reduced body-weight. The difference between the averages for the reduced body-weight is so small in tables V to IX that one would scarcely expect the same rule to hold without exception, and this is noted with few exceptions to be true when a comparison is made between groups with a wider variation in reduced body-weight (tables I to IV).

The variation between males and females (table VII) or between operated and control animals of either sex (tables V and VI) in cases of castration (table VIII) or thyroidectomy (table IX) is not sufficiently marked, it seems to me, to justify the conclusion that there is a constant difference due to sex or the experimental conditions here mentioned, in as far as the organs in question are concerned.

Table X, in which are included the averages for the eleven thyroid-

ectomized and the sixty-four normal animals, shows a marked difference in that the absolute weights and the weights per kilo of the thyroidectomized group are much less than those of the normal group especially when we notice that the reduced body-weight was considerably greater in the thyroidectomized animals. This is not due to thyroidectomy because the normal animals used as controls in the same experiment correspond closely. This variation between the two series seems too large to be explained on the basis of a normal

TABLE I.  
*Spayed Females.*

Reduced body-weight, in kilos.	No. of animals.	Gross body-weight, in kilos.	Weight of gastro-intestinal tract plus contents, in gm.	Weight of gastro-intestinal tract, in gm.	Weight of gastro-intestinal contents, in gm.	Reduced body-weight, in kilos.	Weight of gastro-intestinal tract, in gm. per kilo of reduced body-weight.	Weight of gastro-intestinal tract, in gm.
0.787-1.684	6	1.529	334.0	105.0	229.0	1.300	81.0	1
1.778-1.976	4	2.114	354.0	123.0	231.0	1.883	65.0	1
2.018-2.471	6	2.584	422.0	145.0	277.0	2.307	62.0	1
2.554-2.861	5	3.035	437.0	163.0	274.0	2.761	59.0	1

TABLE II.  
*Normal Females.*

0.756-1.798	7	1.710	330.0	106.0	224.0	1.486	72.0	1
1.838-2.028	7	2.175	339.0	106.0	233.0	1.942	55.0	1
2.072-2.167	7	2.378	380.0	123.0	257.0	2.121	58.0	1
2.260-3.030	10	2.698	380.0	129.0	251.0	2.447	53.0	1

TABLE III.  
*Castrated Males.*

1.142-1.572	6	1.651	327.0	100.0	227.0	1.424	71.0	1
1.614-1.876	6	2.087	377.0	114.0	263.0	1.824	63.0	1
1.916-2.195	10	2.346	399.0	128.0	271.0	2.075	62.0	1
2.210-3.338	9	2.873	452.0	161.0	291.0	2.582	62.0	1

TABLE IV.  
*Normal Males.*

1.328-1.632	7	1.706	310.0	102.0	208.0	1.498	68.0	1
1.652-1.805	5	1.973	353.0	120.0	233.0	1.740	68.0	1
1.812-1.978	9	2.165	377.0	111.0	266.0	1.898	59.0	1
2.112-3.009	12	2.628	388.0	130.0	258.0	2.370	55.0	1

TABLE V.

Group.	No. of animals.	Gross body-weight, in kilos.	Weight of gastro-intestinal tract plus contents, in gm.	Weight of gastro-intestinal tract, in gm.	Weight of gastro-intestinal contents, in gm.	Weight of tract per kilo of reduced body-weight.		Weight of contents per kilo of reduced body-weight.		
						In gm.	Per cent.	In gm.	Per cent.	
Control females.....	31	2.284	364.0	122.0	242.0	2.042	60.0	6.0	119.0	11.9
Operated females.....	21	2.300	387.0	134.0	253.0	2.048	65.0	6.5	123.0	12.3

TABLE VI.

Control males.....	33	2.207	363.0	117.0	246.0	1.961	60.0	6.0	125.0	12.5
Astraded males.....	31	2.315	396.0	129.0	267.0	2.048	63.0	6.3	130.0	13.0

TABLE VII.

Castrated and control males....	64	2.259	379.0	123.0	256.0	2.003	61.0	6.1	128.0	12.8
Operated and control females....	52	2.291	373.0	127.0	246.0	2.045	62.0	6.2	126.0	12.6

TABLE VIII.

Control males and females.....	64	2.244	364.0	120.0	244.0	2.000	60.0	6.0	122.0	12.2
Operated males and females.....	52	2.309	392.0	131.0	261.0	2.048	64.0	6.4	128.0	12.8

TABLE IX.

Thyroidectomized males.....	5	2.098	270.0	90.0	180.0	1.918	47.0	4.7	95.0	9.5
Thyroidectomized females.....	6	2.424	338.0	108.0	230.0	2.194	50.0	5.0	105.0	10.5

TABLE X.

Normal males and females.....	64	2.244	364.0	120.0	244.0	2.000	60.0	6.0	122.0	12.2
Thyroidectomized males and females .....	11	2.276	307.0	100.0	207.0	2.069	49.0	4.9	102.0	10.2

variation. As an explanation, however, it appears reasonable that it may have been due to a difference in the food supplied. The thyroidectomized animals and their controls constituted a series which were killed in the spring after having been fed through the winter for the most part on grain, while the sixty-four normal animals with which they are compared in table X were killed in the fall after having been fed through the summer on grass and vegetables.

Joseph concludes that for practical purposes 10 per cent. of the body-weight of a rabbit should be allowed for the stomach and cecum contents. It will be noted that this percentage agrees with the corresponding percentages in this paper as seen in columns V to X, when it is kept in mind that in the present case the contents of the whole gastro-intestinal tract are included.

According to tables I to IV, which I believe is the more logical grouping, the smaller animals show a larger weight per kilo of body-weight for the gastro-intestinal contents and gastro-intestinal tract than do the larger animals, which is also in accord with Joseph's results.

## ON THE NATURE OF THE OPSONIC SUBSTANCES OF NORMAL SERA.\*

By HANS ZINSSER, M.D., AND EDWARD G. CARY.

(From Stanford University, California, and the Bacteriological Laboratory of Columbia University, New York.)

### PLATE 40.

In spite of the many investigations upon the phagocytosis-enhancing substances of normal and immune sera, there is still much difference of opinion regarding the nature of the antibodies by which these effects are brought about; and the lack of agreement has centered chiefly upon the question of whether the opsonins are separate antibodies, independent of those previously known, or whether opsonic effects can be attributed to the activities of the alexin and sensitizer, acting individually or in coöperation.

Investigations into the nature of the opsonins of normal sera have pointed, with increasing probability, toward the participation of alexin or complement in the opsonic effect. In their earliest communications Wright and Douglas (1) noted the heat sensitiveness of normal opsonins, and since then many other similarities between the opsonic and alexin constituents of the serum have been observed.

The experiments of Hektoen and Roediger (2) showed that, like complements, opsonins are inhibited ("neutralized or bound") by various salt solutions and by formalin.

The work of Lebaditi and Inmann (3) brought out a number of points of similarity which may be tabulated as follows: (a) both alexin and opsonin may be unspecifically absorbed out of serum by normal bacteria; (b) both are absorbed by cellular detritus or organ emulsions; (c) neither is present normally in aqueous humor, but both appear proportionally, with equal rapidity, two or three hours after puncture of the eye; (e) in edematous fluids there is a quantitative parallelism between the two.

Muir and Martin (4) further showed that opsonin, as well as complement, is fixed by specific precipitates and sensitized red blood cells.

It seems fairly clear in consequence of these and other investigations that the substances which exert opsonic action and those which

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activate sensitized complexes cannot be definitely separated from each other by any of our present methods. Nevertheless the prevailing opinion seems to be that the alexin is an important participant in normal opsonic action, but that the process itself is dependent upon a complex mechanism in which normal amboceptor or sensitizer coöperates with the alexin, just as this is supposed to place in the bactericidal or occasional hemolytic effects of normal sera.

This is the opinion of Dean (5), who believes that the mechanism of normal and of immune opsonic action is, in principle, the same, but that in normal sera the amount of sensitizer is so small that the destruction of the alexin reduces opsonic activity to a degree which is too slight to be measurable. Neufeld in a recent treatise expresses his opinion unqualifiedly as follows: "The plasmatic antibodies of normal and immune sera may be divided into two classes: first, the 'tropins,' which are simply constituted substances, do not require coöperation of complement and, therefore, are still active after serum has been heated; and second, the complex 'opsonins,' in the case of which, just as in bacteriolysis and haemolysis, a coöperation of amboceptor and complement is involved."

We shall not discuss differences of opinion regarding immune opsonins and bacteriotropins. As far as the normal opsonins are concerned, the views of Dean and Neufeld are apparently shared by Levaditi and Inmann, Cowdry and Chapin (7), and a number of other investigators, all of whom succeeded in showing that the opsonic power of normal serum destroyed by inactivation can be restored, in part, by the addition of diluted normal serum, too slight in amount to produce powerful phagocytosis by itself.

An analysis of the opsonic properties of the alexin fractions produced by the methods of Ferrata, Sachs-Altmann, and others was therefore undertaken. After the work was well under way it was found that a similar investigation, previously overlooked, had been carried out by Hata (8) in 1908.

Hata found that, as in the case of complement, opsonin is divided by dilution into two parts, neither of which is active alone, but together in proper amounts opsonic action can be restored. It is important to note, however, that the reconstructed opsonin was not, in his experiments, as strong as the original undiluted substance. This, then, draws a complete parallel between alexin or complement and opsonin, as analyzed by the method of complement-splitting. Our results at this time, however, had already begun to show definite discrepancies from those obtained by Hata, and we continued and repeated experiments in order to rule out accidental causes and to arrive at a definite result.

We shall now describe certain general features of our technique which were employed in all the experiments, in order that we may omit in the protocols all but the particular controls which have a definite bearing on the individual experiment.

## METHODS EMPLOYED IN ALEXIN-SPLITTING AND IN THE CONTROL OF HEMOLYTIC ACTIVITIES.

In the preliminary experiments rabbit serum was occasionally used. Fresh normal guinea pig serum was used throughout our later experiments and in the work reported in this paper. The plan of procedure was to separate the alexin or complement into its fractions by various methods, and then to test these split products for both alexin and for opsonic effects, alone and reunited, in parallel series.

We employed for the purpose of alexin-splitting the dialysis method of Ferrata, the dilution method of Sachs and Altmann (dilution with slightly acidified distilled water), and the passage of carbon dioxide through the diluted serum, as advised by Liefmann and others.

In each experiment we determined that each fraction alone was completely inactive in hemolyzing sensitized cells, and that when put together activity was restored. This was done both immediately after accomplished splitting, and again at the time at which the opsonic tests were set up, in order to control possible deterioration of the globulin fraction, which is known to proceed rapidly when this constituent is kept for a few hours dissolved in salt solution.

The error which is most difficult to avoid in the experiments consists in an incomplete separation of the two alexin fractions. We shall not at present go into the work of Bronfenbrenner and Noguchi (9) which throws doubt upon the actual existence of two separate alexin fractions. This will be considered in connection with later experiments. However this may be, it is unquestionably difficult to obtain either an albumen or a globulin fraction devoid of traces of hemolytic activity by any of these methods, and apparently pure fractions may often be shown to be hemolytically active if tested, in sufficient quantity, with highly sensitized cells.

Hence the sensitized red cells which were used for control (sheep cells and anti-sheep rabbit serum) were saturated with sensitizer by the addition of an excess of immune serum, and subsequently washed. In consequence they formed an extremely delicate indicator of hemolytic action, and in the control tests the alexin fractions were added in quantities which corresponded often to many multiples of the amounts powerfully active when the two elements were added together. With a control as rigid as this, we encountered many difficulties in completely splitting the complement. Both the carbon dioxide method and the Sachs-Altmann method of dilution usually yielded a globulin fraction which was inactive, though the albumen fraction in these cases was often slightly hemolytic. For this reason in many of our earlier experiments we combined the carbon dioxide globulin fraction with the dialysis albumen fraction, or end-piece. In later experiments we used the dialysis method entirely, as being likely to produce the least chemical alteration in reaction, etc.

In carrying out the dialysis method our final procedure was as follows: Amounts of 5 to 10 c.c. of fresh guinea pig serum were divided into two parts,

one part being preserved under the same conditions of light and temperature as the dialyzed part in order that it might be used as a normal complement control. The other half was diluted with two parts of distilled water and placed into a fish bladder dialyzing bag which was hung into a jar of distilled water. In this jar a steady trickle of distilled water was allowed to drip out of a five-gallon demijohn, the water being allowed to splash close to the dialyzing bag in order to establish currents; it was removed by siphon arrangement which maintained the water from the bottom of the jar. In this way a constant level was maintained, and from 5 to 10 gallons of distilled water were run through during the course of from 24 to 36 hours. After 24 hours small quantities were removed from the bag, centrifugalized and, after being rendered isotonic with 10 per cent salt solution, were tested against sensitized cells. When the albumen fraction or end-piece, in amounts equivalent to about 0.3 c.c. of the original serum, had been found to be entirely inactive, the bulk of the serum was taken out of the bag and centrifugalized until the supernatant fluid was clear. This was pipetted away and rendered isotonic with 10 per cent. salt solution, constituting our albumen fraction (end-piece). The globulin fraction was dissolved in an amount of salt solution equivalent to the volume of the original serum it represented. It is noteworthy that the globulin fraction so prepared was never in any of our experiments entirely inactive if used in sufficient quantity with sensitized cells. In the majority of cases a rapid hemolysis occurred even when this globulin fraction (mid-piece) was used in quantities equivalent to 0.1 c.c. of the original serum. For this reason it was always reprecipitated by adding distilled water and again dissolved in salt solution. One reprecipitation rarely sufficed, and in some cases there was slight hemolytic activity even after two or three reprecipitations. Although reprecipitation always involved a certain amount of loss, it was continued in every case until the mid-piece, dissolved in salt solution, was found to be entirely inactive. The persistence of hemolytic properties in the dialyzed globulin fraction after precipitation is in itself an interesting phenomenon in that it indicates how slight may be the amount of albumen fraction (end-piece) still capable of hemolyzing in the presence of the globulin, before complete separation has been attained. It seems hardly credible that after two precipitations enough end-piece can be still mechanically adherent to the globulin to cause hemolysis, especially since after complete separation the amount of albumen fraction necessary for activation of mid-piece rarely amounts to less than the equivalent of from 0.025 to 0.05 of the original serum. While no definite explanation can be offered for this peculiarity, it seems to point to the fact that in the original serum the fractions are present as a complex and not separately.

Incidentally in carrying out the preliminary titrations we were able to confirm a number of the observations of Hecker (10) concerning the inactivation which results if the globulin and albumen fractions are allowed to stand together for any length of time. If the two were allowed to stand at room temperature for from two to five minutes before the cells were added, marked inhibition of hemolysis was the result. In some cases distinct inhibition was induced when

<sup>1</sup> This fact has also been repeatedly encountered by Mr. Maltaner, working in our laboratory.

the albumen portion was first run into the tubes containing the red cells and the globulin fraction immediately after it. If the order was reversed prompt laking resulted.

#### METHODS EMPLOYED IN THE PHAGOCYTIC ESTIMATIONS.

Throughout our experiments twenty-four hour cultures of *Staphylococcus pyogenes aureus* on slightly acid agar were used. Emulsions of this were made in salt solution.

The leucocytes employed were obtained from guinea pigs in the following way: when an experiment was to be set up during the morning, the guinea pigs were injected intraperitoneally with five to ten cubic centimeters of a thin aleuronat emulsion on the preceding evening. When the experiment was delayed until the afternoon a similar injection was made early in the morning. As soon as the alexin fractions had been found inactive alone, and active together in the hemolytic tests, the guinea pig was killed, a small incision made in the abdomen, and ten cubic centimeters or more of citrate solution were run into the peritoneum. By gentle agitation this was made to mix with the exudate in the peritoneal cavity and was taken out with nipple pipettes into centrifuge tubes. By gentle centrifugation the clumps were first removed, the supernatant fluid, consisting of an even leucocytic emulsion, was pipetted off, and the leucocytes were washed in two or three changes of salt solution by slow centrifugation to avoid clumping. They were finally emulsified in salt solution and kept in the incubator for rarely longer than one half to two hours before use. The mixtures of alexin fractions, bacteria and phagocytes, were put into small test tubes (4 by  $\frac{1}{4}$  inches) in which they could be easily shaken up, and were exposed to  $38^{\circ}$  to  $40^{\circ}$  C. in a water bath for from forty-five minutes to an hour. During this period they were shaken up three or four times.

After such incubation the tubes were gently centrifugalized in order that the leucocytes might be thrown down and relatively freed from extracellular bacteria. The supernatant fluid was poured off, the sediment reemulsified in a few drops of salt solution, and this emulsion smeared upon slides,—a separate nipple pipette being used for each tube.

Considerable difficulty was encountered in obtaining well stained

preparations in which no gross errors in counting could be made. Satisfactory accuracy was attained in the earlier experiments by the use of Gram's stain after fixation by heat. By this technique the count could be assumed to be accurate, in that the contrast between bacteria and cells was sharp, but there was poor definition of the cells. In our later experiments we stained the slides in the following way with much more satisfactory results: The dried preparations were fixed in heat, and washed in considerable quantities of tap water, in order to remove the salt crystals. They were then stained with Gram's gentian violet for half a minute, and with iodin for ten seconds. After this they were washed in alcohol, decolorized, and directly from the alcohol were plunged for five to twenty seconds into Jenner's solution.

#### EXPERIMENTAL PART.

The tabulation of experiment I illustrates the plan of earlier experiments in which our results were negative, in that no opsonic action appeared either when the albumen fraction or the globulin fraction was used alone, or when the two were used together in quantities in which they produced the rapid and complete hemolysis of sensitized cells. Negative results were the rule in the first fifteen experiments, and it was our inability to account for the fate of the opsonin during the splitting process that forced us to continue the tests.

#### EXPERIMENT I.

##### *Preliminary Titration of Hemolytic Effects.<sup>2</sup>*

Dialysis globulin fraction: 0.1 c.c. represents 0.1 c.c. of original serum.  
Dialysis albumen fraction: 0.2 c.c. represents 0.1 c.c. of original serum.

1. Sensitized cells 0.5 c.c. + whole complement 0.1 c.c.  
= complete hemolysis in 5 minutes.
2. Sensitized cells 0.5 c.c. + globulin fraction 0.4 c.c.  
= no hemolysis.
3. Sensitized cells 0.5 c.c. + albumen fraction 0.6 c.c.  
= no hemolysis.
4. Sensitized cells 0.5 c.c. + globulin fraction 0.2 c.c. + albumen fraction 0.2 c.c.  
= complete hemolysis in 10 minutes.
5. Sensitized cells + salt solution  
= no hemolysis.

<sup>2</sup> The results in the hemolytic titrations were always checked up after two hours at room temperature, and if end-piece or mid-piece alone showed hemolysis, the experiment was discarded.

*Phagocytosis Estimation.*

		Phagocytic average. (100 cells.)
(a)	Experiment.	
	Bacteria .....	0.2 c.c.
	Whole complement .....	0.1 c.c.
	Leucocytes .....	0.2 c.c.
	Salt solution .....	0.5 c.c. = 20 +
(b)	Bacteria .....	0.2 c.c.
	End-piece (albumen) .....	0.3 c.c.
	Leucocytes .....	0.2 c.c.
	Salt solution .....	0.3 c.c. = 2.1
(c)	Bacteria .....	0.2 c.c.
	End-piece (albumen) .....	0.5 c.c.
	Leucocytes .....	0.2 c.c.
	Salt solution .....	0.1 c.c. = 1.8
(d)	Bacteria .....	0.2 c.c.
	Mid-piece (globulin) .....	0.4 c.c.
	Leucocytes .....	0.2 c.c.
	Salt solution .....	0.2 c.c. = 0.8
(e)	Bacteria .....	0.2 c.c.
	Mid-piece .....	0.2 c.c.
	End-piece .....	0.3 c.c.
	Leucocytes .....	0.2 c.c.
	Salt solution .....	0.1 c.c. = 2.5
(f)	Bacteria .....	0.2 c.c.
	Leucocytes .....	0.2 c.c.
	Salt solution .....	0.6 c.c. = 0.2

In seeking for a possible fault in the technique to account for the complete loss of opsonic effects in the experiments, we were led to modify the procedure in two important points. To carry out completely the analogy between the phagocytosis of bacteria and the hemolysis of red cells, in their relation to complement, it seemed necessary to sensitize the bacteria by exposure to heated normal serum before carrying out the phagocytic experiment. It seemed possible also that with unsensitized as well as with sensitized bacteria there might be an error in our experiments depending on the use of excessive amounts of the globulin fraction (mid-piece), since an excess of this is known to inhibit hemolysis. Accordingly we planned our next experiment with these points in view, testing the opsonic action of the split products alone and together (a) upon normal bacteria, (b) upon normal bacteria treated with globulin

fraction (mid-piece), (c) upon bacteria treated with normal serum (sensitized), and upon sensitized bacteria treated with piece, persensitized to follow the designation of Michaelis.

A few of these experiments follow.<sup>8</sup>

#### EXPERIMENT II.

The purpose of this experiment was to determine whether the previous exposure of bacteria to the globulin fraction rendered them more amenable to action by the albumen fraction than they were when the two fractions added together. In each case preliminary hemolytic titrations were carried as before, and phagocytic experiments were not done until end-piece and piece were found entirely inactive alone, and active together. Experiments which hemolysis appeared with end- or mid-piece alone by the next method were not tabulated.

Dialysis albumen fraction; carbon dioxide globulin fraction.

The bacteria were emulsified in salt solution. Globulin fraction 0.2 c.c. added to 1 c.c. of the emulsion, left together at 37.5° C. for 40 minutes, thrown down in the centrifuge and reemulsified in salt solution.

Experimental procedure.	Phagocytic average		
	Experiment (1913).		July 8.
	July 10.	July 11.	
(a) Bacteria +globulin fraction left 40 min., washed, then added albumen from 0.2 c.c. +leucocytes.....	20.0 +	6.9	
(a') Bacteria +globulin fraction, left 40 min., washed, then added salt solution +leucocytes (control for (a) with same emulsion of bacteria).....	0.43	0.6	
(b) Bacteria: Albumen fraction 0.2 c.c. Leucocytes.....	11.8	6.4	
(c) Bacteria: Globulin fraction 0.1 c.c. Leucocytes.....	2.15	1.2	
(d) Bacteria: Salt solution. Leucocytes.....	0.56	0.8	
(e) Bacteria: Whole complement 0.1 c.c. Leucocytes.....	20.0 +	12.0	

The results of this experiment will be discussed below with those of experiments III and IV.

#### EXPERIMENT III.

The purpose of this experiment was to compare in one experiment the phagocytosis of bacteria treated with heated normal serum (sensitized) to that

<sup>8</sup> The amounts of the albumen and globulin fractions used in the phagocytic experiment in which the two were recombined corresponded to those that gave the most prompt and complete hemolysis.

bacteria treated first with normal serum and then with the globulin complement fraction (persensitized) in the presence and absence of the albumen fraction.

Preliminary hemolytic titrations as before.

An emulsion of staphylococci in salt solution was treated with heated normal guinea pig serum for 30 minutes at 37.5° C. (sensitization). The bacteria were then thrown down in the centrifuge<sup>4</sup> and reemulsified in salt solution. This emulsion was then divided into two parts, A and B. To A was added globulin fraction (mid-piece), 0.2 c.c. (to 1 c.c. of emulsion), and this mixture was left for 30 minutes at 37.5° C. Again the bacteria were thrown down and reemulsified. We now had two staphylococcus emulsions, one treated with heated normal serum (sensitized), the other treated with this and also with mid-piece persensitized). The two were equalized as far as possible and the following experiment was carried out. Since it was of course not possible to equalize exactly the two emulsions, it will be seen that we duplicated some of the controls.

Dialysis albumen fraction; dialysis globulin fraction.

Experiment.	Phagocytic average. July 25, 1923.	
A (1) Bacteria sensitized		
Albumen fraction	=	13.2
Leucocytes		
A (2) Bacteria sensitized		
Salt solution	=	1.5
Leucocytes		
A (3) Bacteria persensitized		
Albumen fraction	=	12.6
Leucocytes		
A (4) Bacteria persensitized		
Salt solution	=	2.22
Leucocytes		
B (1) Bacteria sensitized		
Albumen fraction	=	12.7
Leucocytes		
B (2) Bacteria sensitized		
Globulin fraction	=	2.44
Leucocytes		
B (3) Bacteria sensitized		
Globulin fraction	=	9.0
Albumen fraction		
Leucocytes		
B (4) Bacteria sensitized		
Whole complement	=	20.0 +
Leucocytes		

<sup>4</sup> In the centrifugations there was always a loss of bacteria, since they were never completely thrown down within thirty minutes by the centrifuge that we used.

## EXPERIMENT IV.

July 26, 1913.

The purpose of this experiment was to compare normal, sensitized, and sensitized bacteria under the same conditions.

Hemolytic titrations were carried out as before.

Dialysis albumen fraction; carbon dioxide globulin fraction.

Substances added in phagocytic experiment (volumes equalized with salt solution).	Phagocytic average.		
	A. Normal bacteria.	B. Bacteria sensitized with normal heated serum.	C. Bacteria with heat- ed serum then ex- posed to globulin (per cent)
1. Albumen fraction alone.....	9.0	9.8	15
2. Globulin fraction alone.....	0.5	—	—
3. Albumen fraction Globulin fraction.....	16.7	15.3	—
4. Salt solution.....	0.48	1.12	—
5. Whole complement.....	14.0	18.0	—

The results of these experiments indicate that the albumen fraction (end-piece) is the essential factor in determining normal opsonic action. From experiment II (*c*) (three separate measurements) it is seen that mid-piece alone may carry some slight opsonic properties, and, from a comparison of (*a*), (*b*), and (*e*) of the same experiment, that a coöperation of albumen and globulin fractions is more powerfully active than the albumen fraction alone. However, prolonged experience with experiments of this kind leads us to little confidence in the significance of such slight differences in measurement, and we feel that the only positive conclusion that we can draw from experiment II is that bacteria may be powerfully phagocytized in the presence of end-piece or albumen fraction under conditions in which this fraction has absolutely no action on sensitized red cells. The same applies to experiments III and IV. Here again it is suggested that the sensitization of bacteria with heated serum and preliminary treatment with the globulin fraction may reënforce the opsonic action of the albumen portion. E.

<sup>5</sup> 15.1 is the count made with a different emulsion equalized optically and used in the experiments with the normal bacteria. The discrepancy between this count and the other (9.8) shows that no conclusions can be drawn from the slight differences in degree of phagocytosis when the emulsions are equalized optically instead of representing equal parts of the same emulsion.

ferences that indicate this again are slight and we can draw but one positive conclusion; namely, that the essential participant in the normal opsonic action is the end-piece, or albumen fraction.

In the experiments tabulated, as well as in a number of others, this result was unmistakable and definite. Yet for a long time we were not able to produce these results at will, many experiments showing, like the earlier ones, complete disappearance of the opsonic action after complement-splitting. When opsonic action could be determined at all it resided most powerfully in the end-piece. Never did it reside to more than a very slight degree in the globulin, or mid-piece, alone. When phagocytosis occurred after reconstruction of the complement by reunion of the fractions, it occurred also, and with nearly the same power under the influence of the albumen fraction alone. Often, however, it seemed to disappear entirely and was absent in all the tubes.

This inconsistency of results necessitated prolonged experimentation in which it was endeavored to ascertain the factor that determined our frequent negative results. Repeated hemolytic controls seemed definitely to exclude the possibility of deterioration of the complement during the process of separation as an explanation for this. We were finally led to seek the source of variation in alterations of the reaction of the serum during dialysis.

Liefmann and Stutzer (11) found, in 1910, that in bacteriolytic processes end-piece or albumen fraction alone was necessary for the exercise of the complementary function. On the basis of experiments by Braun (12) and further investigations of Liefmann and John (13) they have retracted this view. Bronfenbrenner and Nagyoguchi (14) in an extensive biochemical study of complement-splitting have come to the conclusion that in hemolysis, also, the end-piece alone is the active element, which, in the process of fractionation, is inactivated by change of reaction. It was their work particularly that caused us to focus our attention upon the reaction of our products, in experiments in which we confined ourselves entirely to the dialysis method. The following is a representative titration, which illustrates our general results; namely, that the albumen fraction obtained after dialysis is distinctly more acid than the original serum. Since the accurate titration of small quantities

is subject to considerable possibility of error, we requested Professor Howe, of the Department of Biological Chemistry, to check up our results. The following titration is one which was done by him both with his own solutions and with those prepared by us.

#### TITRATION OF SERUM.

Fresh guinea pig serum was divided into two parts. One part was on ice; to the other (6 c.c.) 12 c.c. of distilled water were added. The serum was placed in a new fish bladder dialyzing bag and dialyzed for 24 hours usual against running distilled water. Two liters of the same water used for dialysis were kept sealed with paraffin, so that no errors arise from this source. The titrations tabulated were done with  $N/250$  sodium hydrate, prepared by Dr. Howe, with phenolphthalein as an indicator. The dialyzed serum was pipetted out of the bag into a clean test-tube. The bag was washed out with this water, sufficient in amount to bring the total volume to 30 c.c. This was then centrifuged until clear.

#### Titrations.

30 c.c. of distilled water	= 0.4 $N/250$ sodium hydrate
Original serum 1 c.c. }	= 0.25 $N/250$ sodium hydrate
30 c.c. distilled water }	
The alkalinity of the serum to phenolphthalein	= 0.15 $N/250$ sodium hydrate
Albumen dialysate equivalent to 1 c.c. of original serum brought to with distilled water	= 1.6 $N/250$ sodium hydrate
Subtract water	= 0.4
The acidity of the dialysate	= 1.2 $N/250$ acid
Gain in acidity during dialysis	= 1.35 $N/250$ acid.

It was evident therefore that the experiments carried out with albumen fraction alone, both hemolytic and opsonic, were under conditions of reaction different from those done with dialyzed complement. Our variable results could possibly be explained, therefore, on the basis of differences of reaction due to various lots of distilled water used for dialysis, when these experiments were begun in California. There we had used water obtained from three different stills, without recording the source of the water in each case. With the water used in the laboratory at Columbia University, always from the same still,<sup>6</sup> uniformly negative results were obtained until we paid attention to the reaction. This was done in experiments of which the following tabulations are examples.

<sup>6</sup> This water was used in the above titrations.

## EXPERIMENT V.

*Dialysis Albumen Fraction.**Preliminary Hemolytic Titration.*

December 6, 1913.

Whole complement 0.5 c.c. + sensitized cells 0.5 c.c. = complete hemolysis in 5 min.

Albumen fraction alone 0.6 c.c. + sensitized cells = no hemolysis in 24 hrs.

Globulin fraction 0.2 c.c. + sensitized cells = no hemolysis in 24 hrs.

Globulin fraction 0.2 c.c. + albumen 0.3 c.c. + sensitized cells = complete hemolysis in 15 min.

*A. Hemolytic Experiment.*

Albumen fraction (end-piece).	Acid or alkali made isotonic with 10 per cent. sodium chloride. <sup>7</sup>	Sensitized cells.	Salt solution.	Hemolysis.
1. 0.4 c.c. + <i>N</i> /350 hydrochloric acid	0.4 c.c. + 0.5 c.c. + 0.2 c.c.			= very slight hemolysis.
2. 0.4 c.c. + <i>N</i> /350 hydrochloric acid	0.2 c.c. + 0.5 c.c. + 0.4 c.c.			= no hemolysis.
3. 0.4 c.c. + no acid or alkali		+ 0.5 c.c. + 0.6 c.c.		= no hemolysis.
4. 0.4 c.c. + <i>N</i> /350 sodium hydrate	0.2 c.c. + 0.5 c.c. + 0.4 c.c.			= no hemolysis.
5. 0.4 c.c. + <i>N</i> /350 sodium hydrate	0.4 c.c. + 0.5 c.c. + 0.2 c.c.			= no hemolysis.
6. 0.4 c.c. + <i>N</i> /350 sodium hydrate	0.6 c.c. + 0.5 c.c. + 0.0 c.c.			= no hemolysis.

*B. Phagocytic Experiment.*

Albumen fraction (end-piece).	Acid or alkali.	Leucocytic emulsion.	Bacterial emulsion.	Salt so- lution.	Percentage average bacteria.
1. 0.4 c.c. + <i>N</i> /350 hydrochloric acid	0.4 c.c. + 0.3 c.c. + 0.3 c.c. + 0.2 c.c.	= 0.18			12%
2. 0.4 c.c. + <i>N</i> /350 hydrochloric acid	0.2 c.c. + 0.3 c.c. + 0.3 c.c. + 0.4 c.c.	= 0.49			23%
3. 0.4 c.c. ....	+ 0.3 c.c. + 0.3 c.c. + 0.6 c.c.	= 1.36			50%
4. 0.4 c.c. + <i>N</i> /350 sodium hydrate	0.2 c.c. + 0.3 c.c. + 0.3 c.c. + 0.4 c.c.	= 4.9			78%
5. 0.4 c.c. + <i>N</i> /350 sodium hydrate	0.4 c.c. + 0.3 c.c. + 0.3 c.c. + 0.2 c.c.	= 6.6			89%
6. 0.4 c.c. + <i>N</i> /350 sodium hydrate	0.6 c.c. + 0.3 c.c. + 0.3 c.c. + 0.0 c.c.	= 4.5			55%

From experiment V it appears that the alteration of reaction does not activate the hemolytic properties of the albumen fraction upon sensitized cells. This is contrary to the results of Bronfenbrenner and Noguchi. The slight hemolysis in tube A (1) we attributed to the acid, and this we found to be the case, as will be seen in the next experiment. The result in the opsonic experiment, however, showed that a restitution of alkalinity restored opsonic power to the albumen fraction, apparently an optimum being reached with the addition of 0.4 of a cubic centimeter of an *N*/350 sodium hydrate solution to 0.4 of a cubic centimeter of the end-piece.

<sup>7</sup> The acid and alkali solutions were made isotonic with 10 per cent. salt solution.

The table (experiment VI) that follows is similar except that at the same time we carried out an attempted restitution of complement (C) by reunion of the fractions, as in the earliest experiments.

#### EXPERIMENT VI.

##### *Preliminary Hemolytic Titration.*

###### A.

- |  |                        |
|--|------------------------|
| 1. Whole complement 0.5 c.c. + sensitized cells 0.5 c.c.                     | = hemolysis in 8 m.    |
| 2. Albumen fraction alone 1 c.c. + sensitized cells                          | = no hemolysis in 2 m. |
| 3. Albumen fraction alone 3 c.c. + sensitized cells                          | = no hemolysis in 2 m. |
| 4. Globulin fraction alone 0.4 c.c. + sensitized cells                       | = no hemolysis in 2 m. |
| 5. Globulin fraction 0.2 c.c. + albumen fraction 0.3 c.c. + sensitized cells | = hemolysis in 20 m.   |
| 6. Globulin fraction 0.3 c.c. + albumen fraction 0.5 c.c. + sensitized cells | = hemolysis in 10 m.   |

###### B.

Results

Serum constituents.	Leucocytic emulsion.	Bacterial emulsion.	Salt solution.	Phagocytic average.
1. Whole complement	0.1 c.c. + 0.3 c.c. +	0.3 c.c. + 1.0 c.c.	= 3.25	
2. Salt solution	1.1 c.c. + 0.3 c.c. +	0.3 c.c. + 0.0 c.c.	= 0.5	
3. Globulin fraction (mid-piece)	0.4 c.c. + 0.3 c.c. +	0.3 c.c. + 0.7 c.c.	= 0.36	
4. Globulin fraction (mid-piece) Albumen fraction (end-piece)	0.3 c.c. + 0.3 c.c. +	0.3 c.c. + 0.3 c.c.	= 0.5	

##### *Hemolytic Experiment.*

###### C.

Albumen fraction (end-piece).	Acid or alkali.	Sensitized cells.	Salt solution.	Result
1. 0.5 c.c. + N/350 hydrochloric acid	0.6 c.c. + 0.5 c.c. + 0.0 c.c.	= hemolysis		
2. 0.5 c.c. + N/350 hydrochloric acid	0.4 c.c. + 0.5 c.c. + 0.2 c.c.	= very slight		
3. 0.5 c.c. + N/350 hydrochloric acid	0.2 c.c. + 0.5 c.c. + 0.4 c.c.	= no hemolysis		
4. 0.5 c.c. + N/350 hydrochloric acid	0.0 c.c. + 0.5 c.c. + 0.6 c.c.	= no hemolysis		
5. 0.5 c.c. + N/350 sodium hydrate	0.2 c.c. + 0.5 c.c. + 0.4 c.c.	= no hemolysis		
6. 0.5 c.c. + N/350 sodium hydrate	0.4 c.c. + 0.5 c.c. + 0.2 c.c.	= no hemolysis		
7. 0.5 c.c. + N/350 sodium hydrate	0.6 c.c. + 0.5 c.c. + 0.0 c.c.	= no hemolysis		
8. Control—acid alone	0.6 c.c. + 0.5 c.c. + 0.0 c.c.	= strong hemolysis in 12 hrs.		
9. Control—alkali alone	0.6 c.c. + 0.5 c.c. + 0.0 c.c.	= no hemolysis		

*Phagocytic Experiment.*

D.

Albumen fraction end-piece).	Acid or alkali.	Leucocytic emulsion.	Bacterial emulsion.	Salt solution.	Phago- cytic average.	Percentage of cells containing bacteria.
1. 0.5 c.c.+N/350 hydrochloric acid	0.6 c.c.+0.3 c.c.+0.3 c.c.+0.0 c.c.				0.36	16%
2. 0.5 c.c.+N/350 hydrochloric acid	0.4 c.c.+0.3 c.c.+0.3 c.c.+0.2 c.c.				0.18	14%
3. 0.5 c.c.+N/350 hydrochloric acid	0.2 c.c.+0.3 c.c.+0.3 c.c.+0.4 c.c.				0.32	22%
4. 0.5 c.c.+N/350 hydrochloric acid	0.0 c.c.+0.3 c.c.+0.3 c.c.+0.6 c.c.				0.38	26%
5. 0.5 c.c.+N/350 sodium hydrate	0.2 c.c.+0.3 c.c.+0.3 c.c.+0.4 c.c.				1.98	64%
6. 0.5 c.c.+N/350 sodium hydrate	0.4 c.c.+0.3 c.c.+0.3 c.c.+0.2 c.c.				3.4	80%
7. 0.5 c.c.+N/350 sodium hydrate	0.6 c.c.+0.3 c.c.+0.3 c.c.+0.0 c.c.				4.01	78%

We may summarize this experiment as follows:

A. By dialyzing guinea pig serum against distilled water for a sufficient length of time a supernatant albumen fraction was obtained which by itself had no hemolytic properties even when added in quantities of three cubic centimeters (corresponding to one cubic centimeter of whole serum) to highly sensitized cells. The precipitated globulins (mid-piece) after two reprecipitations were similarly inactive for hemolysis. But together in suitable amounts these two fractions cause prompt hemolysis.

B. Similarly tested for opsonic properties neither of these fractions possessed any phagocytosis-enhancing powers, nor did they show this when put together in amounts in which they were hemolytically active.

C. When the albumen fraction alone was tested for hemolytic properties in a series in which small amounts of acid or alkali had been added, no reactivation, as found by Bronfenbrenner and Noguchi, could be demonstrated, at least in the quantities employed by us (equivalent to 0.16 of original serum).

D. The albumen fraction (end-piece) alone showed definite and progressively increasing opsonic activity as small amounts of alkali were added, approximately restoring the conditions of reaction prevailing in normal guinea pig serum. Figures 1 and 2 illustrate these relations.

## SUMMARY AND CONCLUSIONS.

Our experiments show that the albumen fraction, or end-piece, obtained by the dialysis of normal guinea pig serum possesses defi-

nite opsonic action. This action is often almost equal to that existing in the unfractionated alexin. It is evident, however, only if reaction maintained during the experiments approximates that in the original serum. By the addition of small quantities of a sodium hydrate solution to the dialyzed serum we have been able to bring back opsonic action which was not evident in the same piece if simply rendered isotonic.

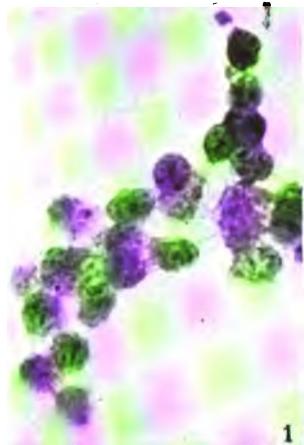
Although our attention was called to the question of reaction by the work of Bronfenbrenner and Noguchi, like Liefmann, we have been unable to reactivate the hemolytic function of end-piece after alteration of reaction. Our experiments suggest that the opsonic action of the albumen fraction is enhanced by preliminary sensitization of the bacteria with heated normal serum and by persons' sensitization of such bacteria with the globulin fraction. However, we cannot be positive of this, since the slight differences of phagocytosis counts upon which such an opinion can be based, fall within the limits of what we consider our experimental error.

The fact that the albumen fraction can exert opsonic activity upon bacteria but cannot hemolyze blood cells seems to us particularly interesting in the light of the fact that alexin can be absorbed by sensitized bacteria but not by similarly untreated blood cells. The literature upon the relation of the alexin fractions to bacteria and the bactericidal effect is confusing in that contradictory results have been obtained by other workers. We are studying this phase of the problem with particular attention to the alkalinity or acidity under which the reactions are carried out.

We think that our experiments do not point to a differentiation of normal opsonin from alexin, but we believe they indicate that the so called end-piece can enter to a slight extent into non-specific relationship with unsensitized bacteria, and it is therefore active, whereas it cannot enter into a similar relation to unsensitized cells. This conception, however, is tentatively made, since we are studying further the non-specific absorption of alexin or complement by sensitized bacteria.

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869.



1



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## EXPLANATION OF PLATE 40.

FIG. 1. Field from preparations in which 0.4 c.c. of the albumen fraction was used with bacteria and leucocytes.

FIG. 2. Same end-piece, or albumen fraction, as figure 1, with same bacteria and leucocytes, with the addition of 0.4 c.c.  $N/350$  sodium hydrate.

## OBSERVATIONS ON A STRAIN OF SPIROCHÆT PALLIDA ISOLATED FROM THE NERVOUS SYSTEM.\*

By HENRY J. NICHOLS, M.D.

Captain, Medical Corps, U. S. Army.

(From the Department of Pathology of the Army Medical School, Washington)

PLATES 41 AND 42.

Our knowledge of *Spirochæta pallida* is now entering on a stage of differentiation of strains, which has already been reached in the study of many other pathogenic microorganisms. In pathology in general, the single, original organism is soon replaced by a group of related organisms and this group is developed as a result of investigation in two directions. On the one hand relationships are discovered with outlying organisms, and on the other hand the original organism itself is resolved into a number of varieties so that the clear cut outlines of our first views give way, on closer observation, to a confusing series of gradations. In the case of *Spirochæta pallida* the same change may be noted. Soon after the discovery of the pallida, an outlying organism was found in *Spirochæta pertenuis*, which, while a distinct organism, undoubtedly belongs in the pallida group (1). On the other hand the pallida itself, according to Noguchi, consists of three different forms, the thick, the thin, and the medium form, and, what is most significant, the thick form produces characteristic lesions in the rabbit, different from those produced by the thinner forms (2).

While the work of differentiating strains is usually confined to biological characters, the explanation of clinical variations is the goal of scientific medicine. From the clinical side there has come a strong suggestion of the existence of strains of pallida, especially of a neurotropic strain. Clinical evidence for this has been cited by many authors (3). Experience has shown

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clinical suggestions based on the observations of the patient alone are often fallacious on account of the many factors involved, but at the same time they form the starting point for experimental work. In the case of some organisms, the typhoid bacillus for example, the lack of a suitable experimental animal has prevented a satisfactory demonstration of the existence of mild or virulent strains, etc. In experimental syphilis, however, we have in the rabbit an animal by which, I think, certain strains can be clearly differentiated.

In this paper I propose to give some evidence on which I believe it is possible to differentiate a strain isolated from the nervous system. Whether the strain should be called neurotropic or simply highly invasive will be discussed later. As far as it is known no observations have been made on a similar strain. Most of the work in experimental syphilis has been carried out with strains isolated from chancres or mucous patches, and the clinical potentialities of these strains are entirely unknown (4). Hence it is not safe to draw clinical conclusions from the lesions in the animal. Again in the only other instance, before ours, in which a strain has been isolated from the nervous system, the experimenter simply made the statement that a typical lesion was produced in one transfer (Hoffmann (5)). Since we isolated this strain, another one has been secured by Uhlenhuth and Mulzer under similar circumstances, but no detailed report on it has been made (6). The results of experimental work in paresis will be referred to later, but it may be said here that no definite conclusions can yet be drawn except that it is difficult to transfer and to continue the strain in the rabbit. If there is any special strain of pallida, the proper way to study it would seem to be to work back from the clinical condition to the experimental animal and to carry on the strain long enough to determine its peculiar characteristics. The subject of strains of pathogenic microorganisms is, of course, a complicated one, as it calls for consideration of variations in both the host and the parasite. In this paper only variations in the parasite are considered and the conditions of the host have been made as uniform as possible by carrying the strain through a large series of animals. Fourteen transfers have been made covering a period of eighteen months, and lesions have been studied in twenty animals. Certain con-

stant characteristics have been noted and these have been compared with lesions developing in four other strains isolated from mucous patches or chancres, one of which has been carried on for four years (7).

The strain in question was isolated by Dr. Hough and myself from the spinal fluid of a case of nervous relapse following salvarsan in the secondary stage of the disease (8). The patient had definite primary and secondary symptoms, and pains in the muscles and joints probably indicated that the nervous system was already involved (9). Two intravenous injections of salvarsan were given and the symptoms rapidly cleared up. Five months later the patient developed a definite psychosis with partial paralysis of the right side of the body. The cells in the spinal fluid reached 840 cubic millimeter and the organism was recovered by injecting three cubic centimeters of whole spinal fluid into a rabbit's testicle. This case was a typical one of nervous relapse in the secondary stage following inadequate treatment. According to our theory of the condition, the nervous system must have been already invaded during the early secondary period, and the patient would probably have suffered from nervous syphilis later.

In morphology the strain corresponds to Noguchi's thick type. While characteristic of the pallida in its sharp regular twists, the body is distinctly thicker than that of many other specimens which have been examined. As a result the curves are not so deep as in the thinner varieties. In Levaditi sections the thickness is especially noticeable, being almost like that of the coarser spirochaetes such as *Spirochaeta refringens*. It is recognized that in dealing with such a minute organism as the pallida, too much weight should not be given to slight differences in form, but a constant difference is of some significance especially when accompanied by distinct lesions, as in the present instance.

Noguchi has noted that on inoculation the thick form produces hard nodules almost cartilaginous in consistence and sharply defined from the surrounding tissues. This description is suggestive in considering the present strain. On inoculation of the scrotum or testicle, a hard, discrete nodule develops and the rim retains this character while the center becomes distinctly necrotic (fig.

Spirochætes are found in abundance in the rim but are scarce in the center of the lesion. The necrosis of the core is not due to fixed infection as no extraneous organism can be demonstrated by cultures. The sort of lesion developed by this strain is easily differentiated from the diffuse, edematous lesion which is most frequently produced by inoculation from chancres and mucous patches. With a medium type of organism, which has been carried on for four years, the lesion always grades off into the surrounding testicular tissue. On intravenous inoculation the same sort of hard, discrete lesion is produced in the testicle and scrotum. In connection with the hardness of the nodule, it is suggestive that nervous relapses have been most frequently noted in patients who have shown the papular form of secondary eruption rather than the acular (10). This clinical finding is probably to be explained by the strain of spirochæta concerned. Histologically the rim consists of a dense collection of lymphoid cells, while the center is composed of a loose connective tissue with few cells and shows evidences of degeneration.

Aside from a characteristic lesion, a characteristic location has been noted, namely, on the scrotal side of the tunica vaginalis. The lesion might be called an internal chancre. The skin surface of the scrotum and the testicle may not be at all affected, but a definite nodule will develop between them. This lesion has resulted both from local inoculation of the testicle and from intravenous infection. A similar localization has been noted in experimental syphilis with ordinary strains and may have no significance, but its occurrence is quite constant with this strain.

The third feature about this strain is a short incubation period which averaged eighteen days in testicular or scrotal inoculations. No great emphasis is laid on this fact, except when it is taken in consideration with the other characters. In Noguchi's two thick forms isolated from protracted chancres, the incubation period was five or six weeks.

The fourth and most striking feature is the generalization of this strain with the production of lesions remote from the site of inoculation. Generalization of the infection from a local inoculation has been noted occasionally by Uhlenhuth and Mulzer and

by others. Occasionally the opposite testicle is affected, rarely the eye. With the present strain one half of the animals observed have shown this generalization. The lesions have been chancres in the opposite scrota, nodules in the opposite testicle, but, most noticeable, keratitis and lesions of the eyelid. The average incubation period of the first lesion was fifteen days, and of the secondary lesion forty days. In other words, about one month has elapsed before the generalization has become evident.

#### GENERALIZATION TO THE EYELIDS.

*Rabbit 5/8*.—Nov. 19, 1912. Right testicle; inoculation with material taken in pipette from testicle of rabbit 5/6; 5th transfer.

Dec. 2, 1912. Definite nodule present (13 days).

Jan. 6, 1913. Large nodule present in right testicle. Left scrotum showing small chancre (48 days).

Jan. 13, 1913. Multiple nodules present on the edge of upper and lower lids. Spirochætae numerous (55 days).

*Rabbit 5/26*.—Oct. 22, 1913. Both scrota inoculated with material taken in pipette from nodule of testicle of rabbit 5/24; 13th transfer.

Oct. 31, 1913. Chancre present, right side (9 days).

Nov. 14, 1913. Chancre present, left side (23 days).

Dec. 6, 1913. Both chancres nearly healed; multiple nodules present on skin of eyelids of both eyes. Spirochætae positive (45 days) (figure 2). Spirochætae also present in left epididymus major (45 days). Spirochætae positive.

The developments in rabbit 5/26 are especially striking because no inoculation was made in the testicle. After a simple cutaneous inoculation, which was followed in nine days by a chancre, there developed in forty-five days multiple nodules in the eyelids and a lesion of the testicle, all rich in spirochætae. Generalization of the virus can often be secured by intravenous injections especially after a strain has passed through a series of animals, but generalization following a simple cutaneous inoculation is an unusually striking occurrence, and it has been observed so often with this strain that it must be regarded as characteristic.

#### GENERALIZATION TO THE CORNEA.

*Rabbit 5/9*.—Dec. 10, 1912. Right testicle inoculated with material taken in pipette from testicular lesion of rabbit 5/8; 6th transfer.

Dec. 30, 1912. Nodule positive in epididymus major (20 days).

Jan. 20, 1913. Nodule positive; testicle removed; large nodule with hemorrhage and necrotic center present. No mixed infection.

Feb. 28, 1913. Both eyes show diffuse keratitis with injection of corneal margin and pannus (80 days after inoculation, 39 days after removal of testicle).  
Apr. 2, 1913. Left eye excised. Spirochætae positive in scraping from cornea, present in large numbers in Levaditi section (figure 3).

In intravenous injection in adult animals the same results are easily produced.

#### CHOROIDITIS.

In the course of the examination of the lesions of the cornea, a new lesion of experimental syphilis in the eye has been discovered by Dr. Louis Green, by the use of the ophthalmoscope; namely, a minute choroiditis. The condition has been noted in a number of animals with this strain after local inoculation, either with or without other lesions of the eye. The condition closely resembles one found in the human eye and consists of hemorrhagic points generally diffused in the lower pole of the eye. A detailed report on this lesion will be made elsewhere, but after careful study of a large number of normal eyes there is no doubt about its occurrence. It has never been observed with other strains except after intravenous injections. In one case the choroid was removed and injected into a rabbit's testicle, but no lesion resulted. Treatment with salvarsan does not produce much improvement in the condition.

As a further evidence of the invading power of this strain, it may be mentioned that local inoculation of the anterior chamber of the eye has always resulted positively, while with other strains my results have been slight and uncertain. The occasional occurrence of one of the generalized lesions mentioned might be disregarded, but their occurrence in one half of the animals observed seems to be clear evidence that we are dealing here with a distinct strain. This chief characteristic may be called its invasive power. It might also be called more virulent than other strains, or more pathogenic, but these terms are more indefinite than the one used.

#### PARESIS.

Now that it has been shown that paresis is "parenchymatous" syphilis of the nervous system, it becomes of interest to know the characteristics of the strain, if there is one, which produces this

disease. Unfortunately the strain seems difficult to transfer to rabbits. Noguchi succeeded in 2 out of 36 animals inoculated from 6 cases in producing lesions with spirochætæ. In one case they were present in few numbers in a section, and in one case they were found in a chancre more abundantly, but the second transfer resulted in only a trivial lesion (11). No mention is made of the type of organism. Berger has reported that after prolonged serial transfers he found a few spirochætæ in 3 slight lesions out of 20 inoculated (12). No attempt was made to continue the strain. Hough and I have made 5 inoculations of spinal fluid from 5 cases of paresis, all with negative results. We have made 16 inoculations of brain substance from 8 cases and have had slight success in 3 instances (13). In one case a slight testicular lesion was followed by a marked double keratitis, and inoculation of the cornea resulted in another slight lesion of the testicle with a definite choroiditis. Inoculation of the choroid resulted in a unilateral keratitis in the third generation, but we have been unable to demonstrate spirochætæ in the lesions or to continue the strain. In the other case a small nodule developed in the testicle after thirty-one days, but spirochætæ could not be found and transfers were unsuccessful. The only conclusion to be drawn from this work so far is that the lesions which result from the inoculation of the brain substance in paresis are slight and contain few organisms. This finding is in marked contrast to the well marked lesion, rich in spirochætæ, which develops with the strain isolated from the case of nervous syphilis. The only resemblance between the two strains is the apparent generalization of our first strain from a paretic in the form of keratitis. The natural assumption is, of course, that in paresis the spirochætæ become modified by their long parasitization in the human brain and lose some of their power to thrive elsewhere. In this way an originally highly invasive strain might not appear so invasive after a few transfers.

#### DISCUSSION.

The evidence presented above seems to me to indicate that the strain in question is a distinct one and it is characterized chiefly by its invasive power. No evidence has been found in the rabbit to indicate any especial neurotropic power, and inoculation of a monkey

brain was negative. By highly artificial means, such as the intravenous injection of a rich emulsion of spirochætæ, I have obtained in one case, with a generalized eruption of the skin, some symptoms of involvement of the nervous system and some slight infiltration in the meninges, but my object in general has been to imitate natural conditions as far as possible with the results given above. I believe that these experimental results may have a clinical equivalent. There is some evidence that the nervous system is more resistant to *Spirochæta pallida* than other parts of the body, and only the more active strains may be able to establish themselves there. In other words, the invasive power of the parasite may be of more importance in syphilis of the nervous system than its affinities. An affinity seems to exist in the sexual organs, and all strains might be called genitotropic, as, after intravenous injection, they invariably locate in the sexual organs whether they do elsewhere or not. But when, after local inoculation, the organism leaves the site of predilection and establishes itself in other tissues, a new factor seems to be introduced and this new factor may be called its invasive power.

Variations in this power seem to afford ground for classifying the members of the pallida group in a series of gradations. *Spirochæta pertenuis* must certainly be classed in the pallida group, but its invasive power is much less than that of the pallida. Yaws is known clinically as a non-venereal, non-hereditary, skin disease. While it is a general disease in which the spirochætæ are distributed by the blood, it also spreads by auto-inoculation in the skin. And following the septicemic stage, the organisms appear always to settle in the skin and not to penetrate the other tissues, such as the nervous system. In the rabbit the pertenuis produces a constant and characteristic lesion of the scrotum consisting of a large, edematous chancre-like lesion which is distinct from the ordinary chancres seen in experimental syphilis (figure 4). On intravenous injections, lesions of the skin of the head are produced which are also distinctive (14). *Spirochæta pertenuis*, therefore, might be put at one end of the series as affecting chiefly the skin. The strain in question might be put at the other end of the series from a point of invasive power in experimental animals and in man. Other

strains of the pallida might be given an intermediate position according to their clinical and experimental characters. If this idea is correct, it is not unreasonable to imagine that in the course of time, an examination of the type of organism in a chancre may be used for prognosis. In this discussion the condition of the host has been intentionally ignored because it is desired to emphasize the definite character in the parasite. The so called resistance of the host must, however, be considered in the last analysis, as it might lessen or accentuate the original characters of the strain, but it could not entirely change them.

#### SUMMARY.

A strain of *Spirochæta pallida* isolated from the nervous system has shown the following constant characters: (1) thick form; (2) the production of hard, well demarcated lesions with necrotic centers; (3) a characteristic location of the lesion; (4) a short incubation period; (5) a tendency to generalize with lesions of the skin and eye, following local inoculation of the testicle and scrotum.

It is suggested that these characters constitute a definite strain of highly invasive power and that a classification of members of the pallida group may be made in accordance with this power.

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FIG. 1.



FIG. 2.

(Nichols: Observations on *Spirocheta pallida*.)



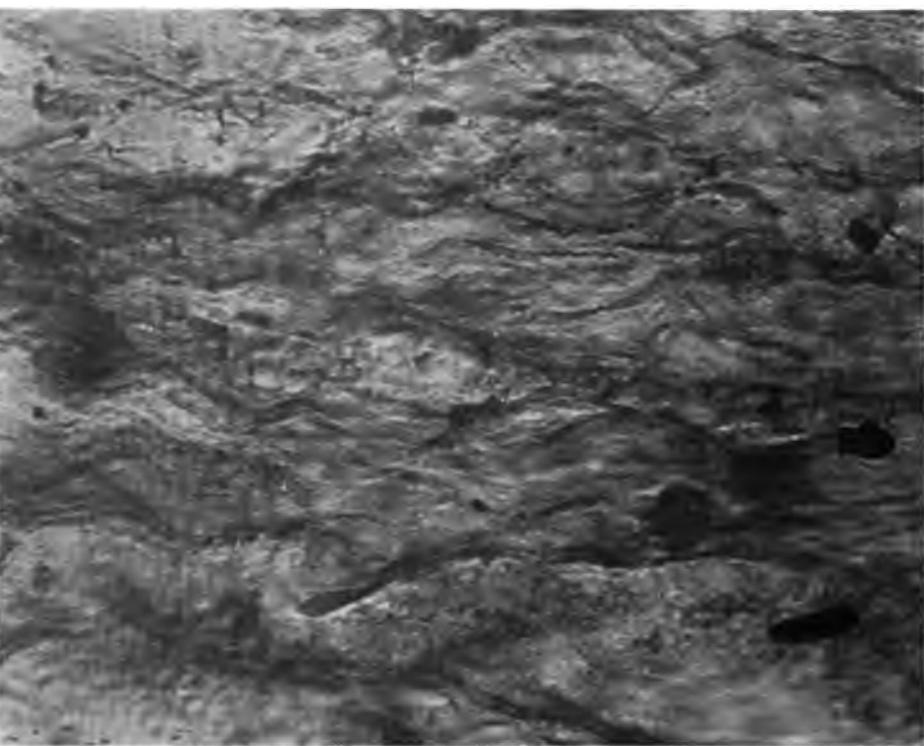


FIG. 3.

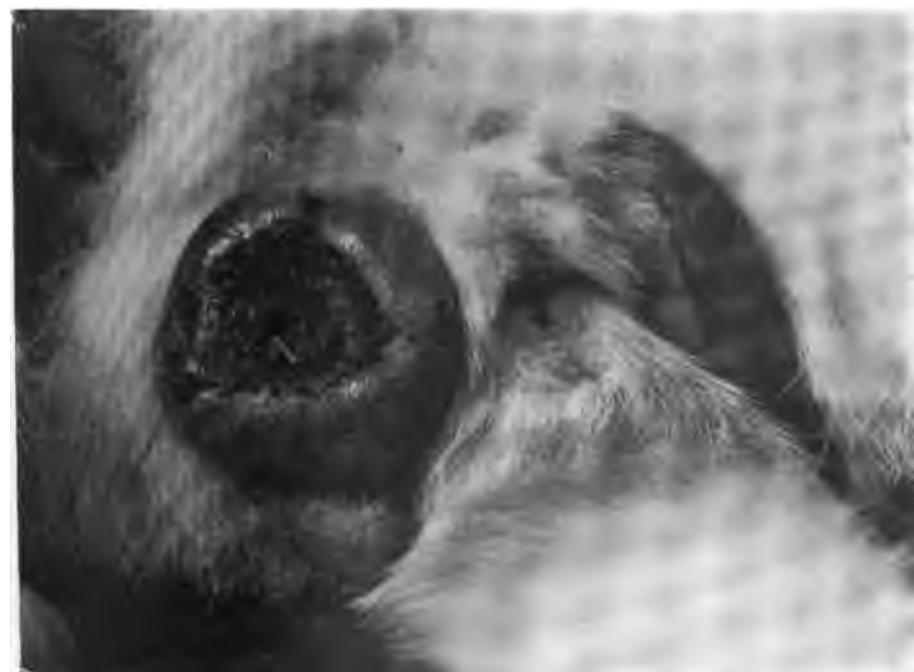


FIG. 4.

(Nichols: Observations on *Spirocheta pallida*.)



EXPLANATION OF PLATES.

PLATE 41.

FIG. 1. Type of lesion produced by subcutaneous inoculation of the scrotum with a strain from the nervous system; hard edge with completely necrotic center.

FIG. 2. Nodules on the margin of the eyelids of rabbit 5/26 (48 days after scrotal inoculation). Spirochætæ numerous.

PLATE 42.

FIG. 3. *Spirocheta pallida* in the cornea of rabbit 5/9; Levaditi section,  $\times 1,300$  (80 days after testicular inoculation).

FIG. 4. Typical lesion of the scrotum produced by subcutaneous inoculation of *Spirocheta pertenuis*; large, edematous lesion; superficial necrosis.

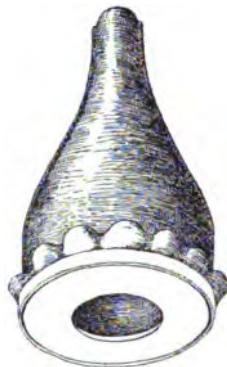
## A NOTE ON NITROUS OXIDE AS AN ANESTHETIC IN ANIMAL EXPERIMENTATION.\*

By DAVID H. DOLLEY, M.D.

(From the Pathological Laboratory of the University of Missouri, Columbia)

As far as the writer can discover from the literature and from personal inquiries, nitrous oxide, now commonly used in surgery, has not been applied as a routine in animal experimentation.

Its use in this laboratory has been limited to the dog, though there is no reason why it can not be extended to other animals. The writer was led to investigate its possibilities in the hope that it would be useful in simple and short operations, such as intravenous administration of drugs for later results, dressings which demand an anesthetic, etc. A test quickly showed that above all it possesses an inimitable value as a preliminary anesthetic, a value



TEXT-FIG. I. Head mask for administration of gas to animals.

so great that it is worth the initial expense for that alone. For a ether anesthesia, a preliminary dose of morphin is apparently inferior to nitrous oxide as regards the ease of handling the animal.

The Gatch nitrous-oxide-ether-oxygen apparatus has been used

\* Received for publication, January 23, 1914.

for administration.<sup>1</sup> It was necessary to devise a suitable head mask, which is represented in the accompanying text-figure. It consists of a cone ten inches in length and five inches in inside diameter at its base, made of fairly thick harness leather. The seams are paraffined inside and out to make it air-tight. The smaller orifice of the cone fits over the orifice of the tube conveying the gases from the apparatus, and the connection again is made air-tight by rubber bushing glued around the inside of the cone's orifice. The tube slips in and out with ease, yet the compressible rubber holds it firmly. The larger orifice is kept distended and circular by a ringlet of brass riveted just inside the opening. A cuff of rubber sheeting is fitted over the larger orifice and has a circular opening for the dog's nose. The cuff is held in place by an outside ring of brass, which slips on tightly. The cuff can therefore be removed from the cone at will, and several with different sizes of opening may be provided to fit the individual case. Cast off automobile inner tubing has been found to combine sufficient strength with the needed elasticity. This contrivance, devised to try out the apparatus, at least offers a tested suggestion for one of better construction and possibly of metal. The important feature is the cuff of rubber, which, with proper size of opening, has been found to fit over a dog's head practically in an air-tight way.

The convenience and ease of handling which the Gatch apparatus promotes may be briefly indicated by a summary of the records on twenty-one dogs. When the cone is applied and the gas turned on, it is slightly more usual for the animal to hold its breath. The average time required from the moment of admitting the gas until the animal becomes perfectly limp was one minute and eight seconds. The maximum time was two minutes and ten seconds, during which the animal held its breath for one minute and ten seconds. The average time of actual inhalation, from the first breath to complete anesthesia, was forty-nine seconds. The maximum duration was one minute and thirty seconds, and the minimum thirty seconds. In only the one instance just mentioned as the maximum has it been over sixty seconds. The time record, however, gives only a partial idea. Without the local irritation of ether and its physiolog-

<sup>1</sup> Gatch, W. D., *Jour. Am. Med. Assn.*, 1910, liv, 775; 1911, lvii, 1593.

ically excitant effect, struggling is reduced to a minimum. In fact many animals do not struggle at all, but, possibly after holding the breath for a few moments, pass quietly to deep inhalations. All the objectionable features of primary ether anesthesia are eliminated. The record embraces dogs of all types, ages, and sizes including five large animals weighing over twenty kilograms. By this method the largest animal has been handled like the smallest one, and readily by two men.

In this apparatus the factor of rebreathing is involved. This question as well as the possibilities of administration are fully discussed in papers by Gatch.<sup>2</sup> It is sufficient to say that with proper management no deleterious effects have been noted from rebreathing. To have three possibilities of instantaneous shift of administration combined within one apparatus, namely, nitrous oxide, ether, and oxygen, is not infrequently advantageous. It seems of value to mention only a few details of application to the dog, as these are for the most part obviously apparent. It has become our practice to turn on the ether as soon as the animal breathes the nitrous oxide deeply. As the animal relaxes the valve box is quickly shifted to in and out breathing of the gas to empty the bag, and is then changed to to and fro rebreathing of ether and oxygen or of ether and air. Air and ether are mentioned because it has been found possible to get essentially the same results as circumstance requires by inflating the bag with air in the place of oxygen by a connection with a foot pump, which is a considerable economy. In either case the shift requires careful management to hold the anesthesia, as the animals come out quickly. Occasionally more nitrous oxide is necessary. Usually the initial bagful of nitrous oxide suffices. If the animal be subjected too long to nitrous oxide alone, fresh air, by disconnecting the tube, and perhaps a compression or two of the chest are sufficient to restore respiration. Our only fatality from this cause was the first experiment. If any objection is raised in regard to an admittedly forced administration of both nitrous oxide and ether at the beginning, it is not a practical one, and at the worst the anoxymia and overconcentration of ether vapor can be no greater than they are by the usual cone method.

<sup>2</sup> Gatch, W. D., *loc. cit.*

The usefulness is not limited to preliminary anesthesia. If desired, as in experiments for recovery, a perfectly even anesthesia with ether may be maintained through the mask, as is proved by records of blood pressure and respiration; or the apparatus may be used only for the preliminary preparation for other ways of administration. It has also been shown to be feasible to maintain anesthesia with nitrous oxide and oxygen. Moreover, the student becomes familiar with the use of an apparatus which is a close imitation of current surgical procedure. Finally, after the initial cost, it is economical. The animal inhales all the ether given, the oxygen can be reserved for an emergency, and, as stated, one bagful of nitrous oxide has been the average per dog.

#### SUMMARY.

Nitrous oxide, administered by means of the Gatch<sup>3</sup> apparatus, as a preliminary anesthetic to ether has been found to be superior for the dog in rapidity of action, in promotion of ease of handling, and in absence of disorder; the anesthesia may be maintained solely by nitrous oxide in combination with oxygen; it is economical; and its use is instructive to students from closer imitation of current surgical methods.

<sup>3</sup> The Gatch apparatus is at present manufactured by the Charles Willms Surgical Instrument Company, Baltimore.

## THE RAPIDITY OF THE INVOLUTION OF ACTIVE THYROID HYPERPLASIAS OF BROOK TROUT FOLLOWING THE USE OF FRESH SEA FISH AS A FOOD.\*

By DAVID MARINE, M.D.

(From the H. K. Cushing Laboratory of Experimental Medicine of Western Reserve University, Cleveland.)

In a recent paper (1) the effect of certain foods on the production and prevention of goitre in brook trout (*Salvelinus fontinalis*) was described and it was shown that a continuous diet consisting only of hog's liver and heart<sup>1</sup> quickly (in one to two months) induces well marked thyroid hypertrophy and hyperplasia, and that when hashed fresh sea fish alone was substituted, further hyperplasia was prevented, and any existing thyroid hyperplasia involuted to its colloid or quiescent state.

In the present communication will be given data concerning the rapidity of the involution following the substitution of the fish diet and the time limits within which it is completed, and also, for comparison, the time limits of the involution induced by other means in Lake Erie pike, in brook trout, and in mammals (dogs).

The fish used in these observations were taken from the regular stock hatched about January 15, 1913, and distributed to the ponds about July 1. They had, therefore, in common with the entire 1913 hatch, been fed with finely divided hog's liver for the first few months (four to five), and after that with a mixture of heart muscle and liver. I arbitrarily chose fish from pond 8,<sup>2</sup> since complete examinations made in August of all the 1913 hatch, both those living in the troughs and those in the ponds, showed approximately the same degree of thyroid hyperplasia.

\* Received for publication, February 7, 1914.

<sup>1</sup> The food is but one of a complex of interacting factors, among which are overcrowding, and relatively insufficient water supply, with its diminished oxygen and excess of excreta contents. The food, however, is believed to be the major single factor.

<sup>2</sup> For the plan of the hatchery see Marine, D., *Jour. Exper. Med.*, 1914, xix, 71.

The experiments were conducted as follows: On September 28, 1913, the food of all the fry of 1913 was changed to fresh butter fish (*Stromateus triacanthus*) given once daily except for the weekly fast day. Eight fish were taken from pond 8 on this date as controls, and four fish were removed every seventh day for sixty-three days following. The data are presented in the following tabulation.

The eight fish taken as controls at the beginning of the experiment have uniformly hyperplastic thyroids listed as "marked hyperplasias," comparable and similar in all essentials to those present in the fry of corresponding ages in 1909, 1910, 1911, and 1912. The strain of fish and the food (hog's liver and heart) have also remained constant during these years. The distribution of the thyroid tissue is approximately the same in all the forty-four fish used. In all cases the thyroid follicles have filled the thyroid area about the aorta and between the 1st and 3d gill arches, have extended up to the pharyngeal mucosa, have invaded the bone and muscles, and in most cases have extended through to the skin adjacent to the ventral attachments of the 1st and 2d gill arches.

Specimens taken seven days after the feeding of fish was instituted show slight but distinct changes in the thyroid toward involution. The follicular spaces are more distinct and contain some stainable colloid. The epithelium is slightly changed, and the stroma and vascularity appear unmodified, and, according to the arbitrary standards I have used, such a histological appearance would be designated "colloid-moderate-marked hyperplasia."

The fourteen day specimens are listed as "colloid-moderate hyperplasias," *i. e.*, the stainable colloid is more abundant, the follicular spaces better defined, and the epithelium has shrunken from high columnar to low columnar.

The twenty-one day specimens show still further accumulations of colloid in the follicles, and the lining epithelium has shrunken to the cuboidal form. These specimens are listed as "colloid-early hyperplasias."

Specimens removed twenty-eight days after the fish feeding was instituted show still further shrinking of the epithelium, and the colloid content is now nearly uniform and homogeneous. Vacuoles

TABLE I.

Pond No.	Age in months.	Date taken.	Food and length of time fed.	Extent of overgrowth.	Condition of thyroid.	Classification.	Remarks.
8	8 +	Sept. 28, 1913	Hog's liver for 1st 4 mos. of life; then hog's liver and heart	Entire subpharyngeal area infiltrated, including bone and muscle	Marked hyperplasia	This is an example of 8 controls. All have approximately the same degree of hyperplasia.* Colloid absent.	
8	8 +	Oct. 5, 1913	Whole butter fish only, beginning Sept. 28, 1913, 1 wk.	Entire subpharyngeal area infiltrated, including bone and muscle	Colloid moderate or a marked hyperplasia (beginning in volu-	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid appears in follicles.	
8	8 +	Oct. 12, 1913	Butter fish only for 2 wks.	Thyroid area filled, extending to pharyngeal mucosa, in bone and muscle	Colloid-moderate hyperplasia. (involuting)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid in fair amounts in all follicles. Epithelium low columnar.	
8	9	Oct. 19, 1913	Butter fish only for 3 wks.	Entire subpharyngeal area filled, extending to pharyngeal mucosa and skin and gills, in bone and muscle	Colloid-early (involuting)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid dense, nearly uniform. Follicles more separate and stroma more prominent, due to shrinkage of blood supply and size of follicles. Epithelium cuboidal.	
8	9 +	Oct. 26, 1913	Butter fish only for 4 wks.	Entire subpharyngeal area filled, extending to pharyngeal mucosa, bone, and muscle	Colloid-early (approxi- mating pure colloid)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid dense, nearly uniform. Follicles more separate and stroma more prominent, due to shrinkage of blood supply and size of follicles. Epithelium cuboidal.	

Pond No.	Age in months,	Date taken.	Food and length of time fed.	Condition of thyroid.		Remarks.
				Extent of overgrowth.	Classification.	
8	9+	Nov. 2, 1913	Butter fish only for 5 wks.	Entire subpharyngeal area filled, extending to pharyngeal mucosa, to bone, muscle, and gills	Colloid goitre (complete involution)	This is an example of 4 specimens examined. All have approximately the same thyroid condition. Colloid dense, uniform. Follicles separate. Epithelium low cuboidal.
8	9+	Nov. 9, 1913	Butter fish only for 6 wks.	Entire subpharyngeal area filled, extending to pharynx mucosa, bone, and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens examined, all showing same thyroid condition.
8	10	Nov. 16, 1913	Butter fish only for 7 wks.	Entire subpharyngeal area filled, extending to skin, pharyngeal mucosa, bone, and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens examined, all showing same thyroid condition.
8	10+	Nov. 23, 1913	Butter fish only for 8 wks.	Entire subpharyngeal area filled, in bone and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens examined, all showing same thyroid condition.
8	10+	Nov. 30, 1913	Butter fish only for 9 wks.	Entire thyroid area filled, extending to pharyngeal mucosa, to bone, and muscle	Colloid goitre (complete involution)	This is an example of 4 specimens, all showing same thyroid condition.

are still present at the colloid-epithelium junction. By the use of less rigid standards than I have adopted, these thyroids could be classified as completely involuted rather than as "colloid-earthyperplasias." By comparing the follicles with those of normal fish or with those seen in the subsequent specimens of this series, however, it is evident that the involution is not complete. The specimens taken after thirty-five days of fish feeding are listed as "complete involutions"; that is, the lining epithelium is low cuboidal. The stainable colloid is homogenous, and nearly uniform in all follicles. The follicles are smaller and more widely separated from each other, while the stroma is correspondingly more prominent. The capillaries about the follicles are less prominent. The distribution of the follicles is, of course, the same as that attained during the growing actively hyperplastic stage, *i. e.*, in bone and muscle with extension to the skin and pharyngeal mucosa.

The specimens examined on the 42d, 49th, 56th, and 63d day respectively are nearly identical with those of the 35th day. There is probably some further involution in the last specimens, as evidenced by a slight increase in the staining density of the colloid and in the flattening of the lining epithelium. This is also true of normal thyroid follicles, and has occasioned much discussion as to what is the normal type. In a tissue like the thyroid epithelium, which is capable of such marked hypertrophy and hyperplasia, as well as marked involution, one must adopt somewhat arbitrary groups within which variations between certain narrow limits are not taken into account.

The time in which complete involution of these relatively mild degrees of thyroid hyperplasia occurs, following the use of butter fish in this particular environment and strain of brook trout, is about thirty-five days. As was shown in a previous paper (1), the thyroid gland, once involuted, remains in this colloid or involuted stage as long as the fish are fed with this food. No further growth of the thyroid takes place under these conditions. Thus the thyroids of the three and two year old brook trout at this hatchery have the same amount of thyroid tissue with the same distribution of follicles that was attained during the first ten months of their lives, at which time the change of food was instituted.

Iodin involutes the hyperplasias more rapidly than butter fish. In Lake Erie pike with mild degrees of thyroid hyperplasia it was found (2) that iodin involuted them in from sixteen to eighteen days.

In the experiments of 1909 (3) it was found that the thyroid of twenty months old brook trout with extensive hyperplasia involuted in from twenty to twenty-five days following the daily addition of traces of iodin to the water. In the iodin experiments of 1910 (4) it was found that involution took place in about forty days in twenty-nine months old brook trout with large and often slightly ulcerated goitres, while in forty-one months old emaciated brook trout with extensively infected and ulcerated external goitres healing and involution were complete at the sixty-second day. In mammals the involution with iodin is of course more rapid (5). In young dogs with uncomplicated simple hyperplasias, the involution is usually complete in from fourteen to eighteen days.

As the whole butter fish contains appreciable amounts of iodin<sup>4</sup> one may ask whether this effect of fish as food is merely the well known iodin action, and the somewhat longer involution time only the manifestation of a very dilute solution. In the absence of any data against this view, I prefer tentatively to consider it an iodin effect rather than to postulate another explanation in favor of which there are at present no direct data. Experiments are now in progress which it is hoped will shed some light on this question.

\* Iodin in fish, birds, and mammals is, for the most part, contained in the thyroid glands, although traces of iodin have been found in certain fish oils, as, for example, cod liver oil, where presumably contamination with thyroid was avoided. Using the modified Baumann and the Hunter methods, I have not been able to recognize traces of iodin in whole amphioxus and in butter fish (*Stromateus triacanthus*) and weak fish (*Cynoscion regalis*) from which the thyroid areas had been removed. A. T. Cameron (*Biochem. Jour.*, 1913, vii, 466; *Jour. Biol. Chem.*, 1914, xvi, 465) has published the finding of relatively large amounts of iodin in the thyroids of rays and dog fish. Thus in *Raja clavata* he found the mean iodin content to be 0.438 per cent. of dried gland, while in *Scyllium canicula* he found a mean content of 1.16 per cent. of dried gland. I have made the following iodin estimations in butter fish:

- (1) With whole butter fish in 1 gm. amounts = possible trace.
- (2) With whole butter fish in 5 gm. amounts = trace, unmeasurable.
- (3) With butter fish, exclusive of the thyroid area, in 1 gm amounts = no trace.
- (4) With butter fish, exclusive of the thyroid area, in 5 gm. amounts = no trace.

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# A STUDY OF THE FERMENTS AND FERMENT-INHIBITING SUBSTANCES IN TUBERCULOUS CASEOUS MATERIAL.

## STUDIES ON FERMENT ACTION. XII.\*

By JAMES W. JOBLING, M.D., AND WILLIAM PETERSEN, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons, Columbia University, New York.)

In a previous paper (1) we reported that sodium soaps prepared from ether-soluble extractives of tubercle bacilli inhibit the action of trypsin and leucoprotease. The inhibiting action was found to depend on the presence of soaps of the unsaturated fatty acids, and could be destroyed by saturating the acids with iodin.

The results of this study appear to have a direct bearing on the development of caseation in tuberculosis. Caseation in tuberculosis is a form of coagulation necrosis in which the dead tissues rarely undergo autolysis, except as a result of secondary infection. Syphilis is the only infectious disease presenting a similar condition. In other instances of coagulation necrosis the dead tissues are soon removed by means of autolysis and phagocytosis. It appears, then, that substances having the property of preventing autolysis must be present in syphilitic and tuberculous tissues.

This view is confirmed by the work of Schmoll (2) who was unable to find any evidences of autolysis in caseous material. The results obtained by Auclair (3) can be explained in a similar manner. Auclair believes that caseation is due to specific toxins of the nature of fats which are soluble in ether, chloroform, and benzin. These extracts suspended in water produced typical caseous abscesses when injected into the subcutaneous tissues of animals, and caseous areas in the lungs when injected into the trachea of guinea pigs. No doubt the conditions observed by Auclair closely simulated caseous areas, but we doubt if they were due to the presence of specific toxins. It is more probable that his injections caused the usual inflammatory exudate which assumed the character of the caseous matter owing to the failure of autolysis.

The lack of autolysis in caseous material is similar to that observed in non-

\* Received for publication, January 21, 1914.

infected anemic infarcts. In the latter condition the dead tissue is finally moved by the phagocytic leucocytes which invade it from the surrounding tissue. The leucocytes also liberate ferments which aid in causing solution and absorption of the dead tissue. It is only in the large infarcts that softening occurs, this is in the central part of the mass, while the periphery remains firm. Wiesner (4) found that the autolytic enzymes act best in an acid medium, and Wells believes that this explains the central softening in large infarcts. According to Wells, the acids at the periphery are neutralized by the blood plasma, so that autolysis is active only at the center.

Anemia, due to occlusion of the blood vessels, may be an important factor in causing caseation in the chronic forms of tuberculosis. However, in acute caseous pneumonia some other explanation seems necessary. Here we do not have the connective tissue reaction seen in the chronic forms of the disease, and we must therefore look for some other cause to explain the conditions present.

In a previous report (6) we showed that tubercle bacilli contain unsaturated fatty acids which, when saponified, are able to inhibit enzyme action. We believe that this observation explains the lack of autolysis in caseous matter, but it was considered necessary to make a careful study of caseous material in order to find out what ferments and ferment-inhibiting substances were present.

Schmoll's (7) failure to find evidences of autolysis in the caseous material from lymph glands indicates that ferment action has been inhibited from the start. This assumption is based not only upon Schmoll's work, but also upon our observation (8) that trypsin is destroyed when incubated for thirty minutes with soaps of the saturated fatty acids. If this view is correct, failure of proteolysis is due to destruction of the ferments by the soaps, but we must also consider the possibility of undestroyed ferments being present, though inactive, owing to the presence of inhibiting substances. It is unlikely that free ferments should be present in the caseous material obtained from chronic tuberculous lymph glands, but they may be present in a rapidly progressing caseous pneumonia with marked inflammatory exudate. In the latter condition it is impossible to separate the caseous material from the inflammatory exudate, and so extracts of the caseous material will probably show the presence of some undestroyed ferments.

The caseous matter used in this study was obtained from tuberculous lymph glands and from several cases of rapidly progressing

caseous pneumonia.<sup>1</sup> The method used to determine the extent of digestion was that described in our previous reports. The mixture was made definitely acid with a solution composed of 10 per cent. acetic acid and 20 per cent. sodium chloride. The tubes were then placed in boiling water for five to ten minutes to coagulate the protein and the contents afterwards filtered through kaolin. The total incoagulable nitrogen was determined according to the method recommended by Folin.

#### CASEOUS LYMPH GLANDS.

The caseous matter derived from the lymph glands was spread in thin layers on glass plates and dried at temperatures below 45° C. After being dried the material was ground in a mortar to a fine powder. For the experimental work the emulsions were prepared in the proportions of one gram of caseous matter to 100 cubic centimeters of water. The material was examined to find out if ferments and ferment-inhibiting substances were present and also for evidences of autolysis. To determine the extent of autolysis a flask containing fifty cubic centimeters of the emulsion was placed in the incubator and left there for five days. Every day five cubic centimeters were removed and the amount of incoagulable nitrogen was determined. The incoagulable nitrogen obtained in five cubic centimeters of the emulsion on the fifth day was practically the same as that obtained on the first day. The results of this experiment indicate that either ferments are not present in the caseous matter, or their activity is suppressed. The freshly prepared emulsion was neutral in reaction, but became slightly acid after the second day. This is important as in the beginning the reaction was favorable to the ferments active in an alkaline or neutral reaction, and after the second day it was favorable to those active in an acid reaction, yet autolysis did not occur.

We obtained somewhat different results in a similar experiment conducted with caseous material from a lymph gland which had become secondarily infected. Autolysis occurred in a more marked

<sup>1</sup> We wish to thank Dr. F. Mathews of St. Mary's Free Hospital, Dr. Larkin of the New York Hospital, and Dr. Lamb of the Presbyterian Hospital for their kindness in furnishing us with material.

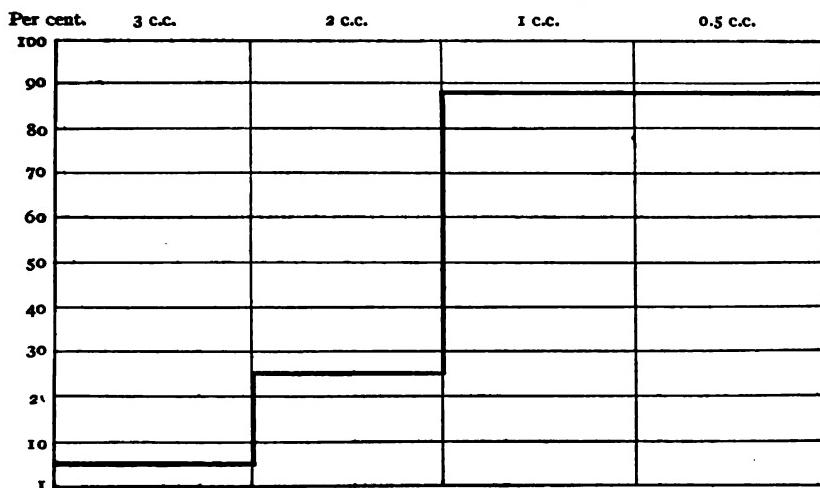
degree in an alkaline reaction, but was much more active in an acid reaction. In no instance have we been able to obtain evidences of autolysis in emulsions of uninfected caseous matter of lymph glands either in acid or alkaline reactions. The lack of autolysis in the caseous material might have been due to the presence of soaps, and we therefore attempted to rule out this factor.

Two methods were used to determine the presence of undestroyed ferments. In the first the whole emulsion was treated with an excess of alcohol to which a small amount of hydrochloric acid had been added. Treated in this manner the ferments and protein were precipitated, and the fatty acids liberated by the hydrochloric acid were made soluble in the acid alcohol. The precipitate was then washed with alcohol and ether, and dried. The powder thus obtained was made into an emulsion with water, a portion of made acid and another alkaline, and placed in the incubator for several days. The presence of ferments would have been shown by the degree of autolysis that occurred. In the second method the emulsion was first incubated for twenty-four hours in an alkaline solution in order to get the ferments and soap into solution. It was then centrifuged and the supernatant fluid treated as was the whole emulsion. In this instance the powder was made into an emulsion and tested against casein. In neither case were we able to demonstrate the presence of ferments.

We believe that the results of these experiments justify us in concluding that undestroyed ferments are rarely present in caseous lymph glands which have not become secondarily infected.

As practically all cells contain intracellular ferments, we must assume that substances were present which prevented autolysis. Our previous work indicated that these substances were probably soaps, therefore our next experiments were planned to see if this was the case. A fresh 1 per cent. emulsion was prepared and kept at room temperature for several hours. During this period it was shaken frequently. It was then centrifuged until it became clear. The supernatant fluid was now removed, made strongly alkaline with sodium hydrate, and the flask containing it placed in boiling water for several minutes. The fatty acids were then liberated with hydrochloric acid, taken up in ether, and resaponified.

The last step was repeated in order to free the soaps from unsaponifiable matter. The soaps were then tested for their enzyme-inhibiting action. They were mixed in various dilutions with trypsin and placed in the incubator at 37° C. for thirty minutes. The casein was then added and the mixture placed in the incubator again for about two hours. After the final incubation the total incoagulable nitrogen was determined according to Folin's method. Text-



TEXT-FIG. I. Effect of the saponified extract from caseous lymph glands on tryptic digestion.

figure I shows the influence of soaps obtained from the caseous matter of lymph glands on the activity of trypsin.

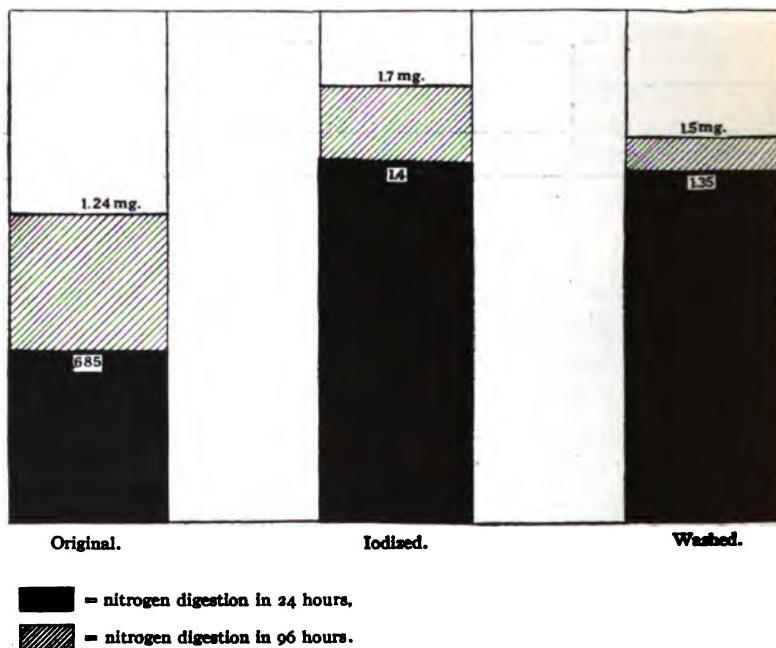
The soaps had been dissolved in sufficient water so that one cubic centimeter of the solution represented the amount of soap contained in one cubic centimeter of the original 1 per cent. emulsion. Three cubic centimeters of the emulsion, representing the soap contained in 0.03 of a gram of caseous matter, was sufficient to inhibit the action of the ferment. In the tube containing two cubic centimeters the digestion was only 25 per cent. of that obtained in the control tube containing no soaps.

The results obtained with the caseous material taken from a lymph gland which had become secondarily infected differ from those just described. Similar experiments were conducted with

this material, which was semifluid in consistence, and ferment active in alkaline and acid reactions were demonstrated. While autolysis occurred in both acid and alkaline reactions, it was more marked in the acid reaction, indicating that soaps were still present.

With this possibility in mind we decided to study the influence of iodin on the whole emulsion to see if it would accelerate autolysis. We have already shown (9) that treating the soaps of the unsaturated fatty acids with iodin destroys their ferment-inhibiting action, and it is therefore of interest to determine if similar results can be obtained with the native mixtures of protein and soaps.

The emulsion was divided into two portions, one of which was used as a control. To the other was added a mixture of iodin and iodide of potassium. The mixture was allowed to stand for several hours, and the excess of iodin was then removed by repeated extractions with chloroform. A third flask containing the washed precipitate was also used. To each of these flasks trypsin was



TEXT-FIG. 2. Effect of iodin on ferment-inhibiting substances present in caseous matter of lymph glands.

added. Text-figure 2 shows the amount of incoagulable nitrogen in two cubic centimeters of the emulsion after twenty-four and ninety-six hours' incubation at 37° C. The first column shows the amount of incoagulable nitrogen obtained after digesting the untreated emulsion with trypsin. The second column represents the amount of nitrogen obtained by digesting the iodized fraction, and the third the action of trypsin on the precipitate which had been freed from soaps by extraction with water and repeated washings.

A study of this chart shows that in the first tube soaps were not present in sufficient quantity to inhibit the action of the trypsin, but that digestion in a similar fraction treated with iodin was as great at the end of twenty-four hours as that obtained in the first tube after ninety-six hours. In the third tube the soaps had evidently been removed to a large extent, though digestion in the last seventy-two hours was not equal to that observed in the iodized fraction.

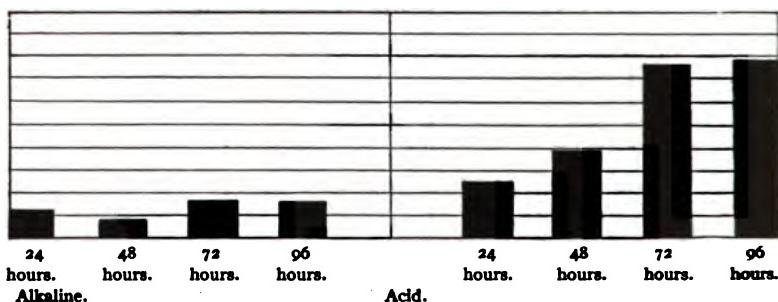
#### CASEOUS PNEUMONIA.

The affected areas in the caseous lungs were freed as much as possible from the less involved lung tissue, cut up in a meat machine, and dried. When dry the material was ground to a fine powder in a mortar. We anticipated somewhat different results in these experiments from those obtained with caseous material from lymph glands, owing to the difficulty of freeing the caseous material from the inflammatory exudate. In the former case there is usually no inflammatory exudate and therefore no extraneous ferments, while in the material from caseous pneumonia these ferments must be considered.

Several specimens of this kind were examined, and practically similar results were obtained with all. As in the experiments conducted with the caseous matter of lymph glands, the emulsion was made in the proportion of one gram of the dried material to one hundred cubic centimeters of water.

In the first experiment we wished to determine the extent of autolysis. Two flasks containing twenty-five cubic centimeters each of the emulsion were incubated for several days at 37° C. In order to determine the type of ferments present, the contents of one of

the flasks was made slightly acid in reaction, and the other slightly alkaline. Text-figure 3 shows the result of this experiment. In studying this chart it should be remembered that soaps are dissociated in an acid reaction, and are therefore inactive as inhibiting agents. Each line on the chart represents 0.1 of a milligram of nitrogen, and the black columns indicate the incoagulable nitrogen



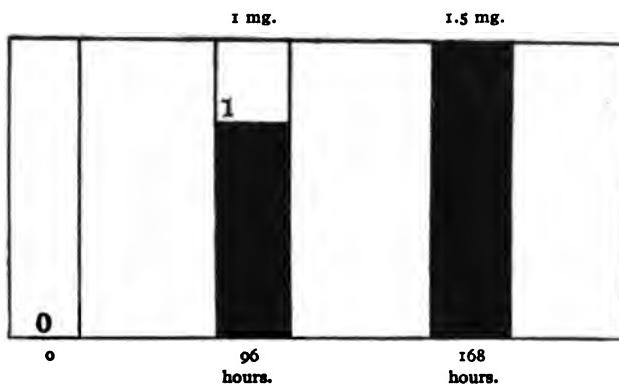
TEXT-FIG. 3. Autolysis of caseous material in acid and alkaline reactions.

two cubic centimeters of the emulsion. The chart shows the differences obtained in the presence of acids and alkalies. Inhibiting substances were present in sufficient quantity to prevent the action of ferment active in an alkaline reaction, but they were not effective when the reaction was made acid.

In some of our preliminary experiments we observed that autolysis occurred in certain of the emulsions which had been made slightly alkaline, and we were at a loss for the proper explanation as soaps were known to be present. We found later that in practically all such instances the reaction of the emulsion had become acid, thus throwing out the soaps and permitting the action of ferment active in an acid reaction. In the last experiment it was evident that ferment active in an acid reaction were present, but that those active in an alkaline reaction were absent or their activity was suppressed by the soaps. In the next experiment we wished to determine if ferment active in an alkaline reaction were also present in the caseous material.

The emulsion was treated with an excess of alcohol which had been made slightly acid. By this means we hoped to precipitate the ferment uninjured and at the same time to remove the soaps, or

rather the fatty acids, which, treated in this manner, are soluble in alcohol. The precipitate was removed by centrifugation, washed with alcohol and ether, and dried. It was then brought up to the original volume with water, made slightly alkaline, and placed in the incubator for seven days. The results of this experiment are seen in text-figure 4. The columns represent the amount of incoag-



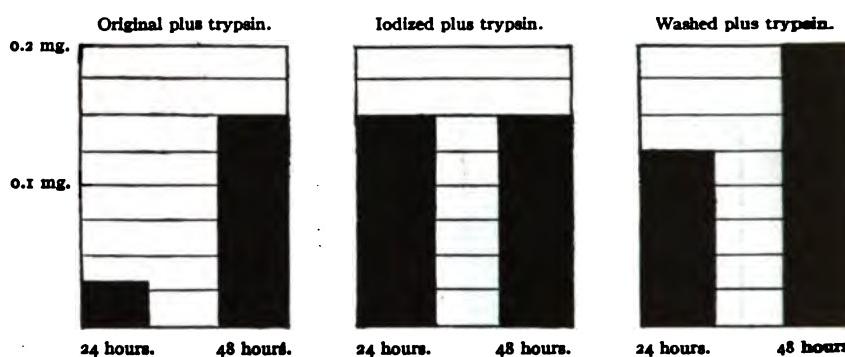
TEXT-FIG. 4. Autolysis of alcohol precipitate.

uble nitrogen in two cubic centimeters of the mixture. The chart shows that ferment active in an alkaline reaction were present in the original material, but that their activity was inhibited by the soaps. When these were removed by the acid alcohol, the caseous matter underwent autolysis. It is unlikely that these tissues become acid during life, and so the lack of autolysis is easily explained.

Soaps were present in smaller amounts than in the caseous matter of lymph glands. This may be due to the fact that the rapidity of the process did not permit an accumulation of the soaps such as probably occurs in the chronic tuberculous lymph glands. Text-figure 3 shows that autolysis did not occur when the original emulsion was made slightly alkaline, though in other experiments in which the soaps were first removed, we demonstrated the presence of ferment active in an alkaline reaction. These results indicate a slight excess of soaps over the amount needed to prevent autolysis. This view is confirmed in the next experiment in which it is shown that with an increase of ferment autolysis ensues.

In our experiments with the caseous matter of lymph glands we

showed that previous treatment of the emulsion with iodin was effective in aiding the action of trypsin. We now made similar experiments with the pneumonic material, using the same technique. Text-figure 5 shows the results obtained. Two controls were used



TEXT-FIG. 5. Effect of iodin on ferment-inhibiting substances in caseous pneumonia.

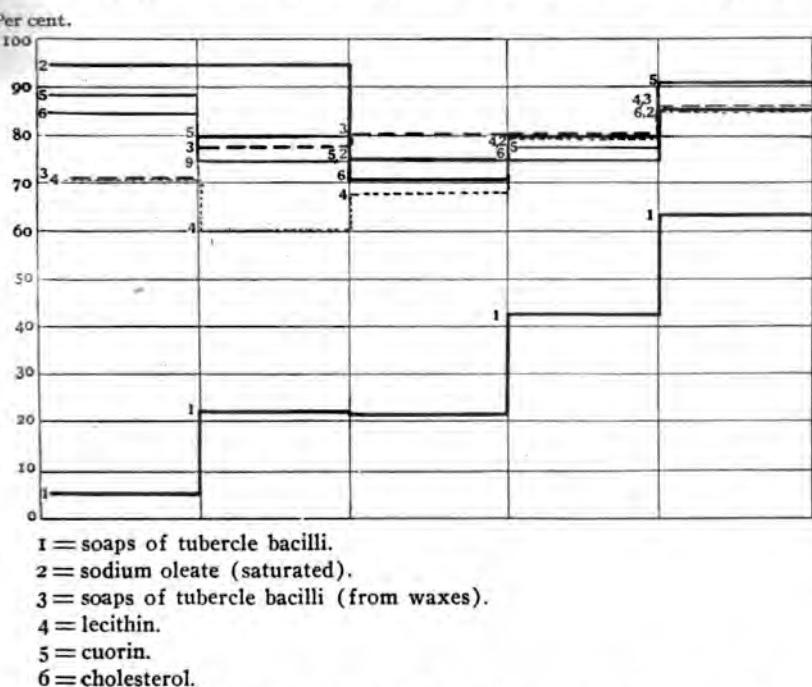
One control consisted of the untreated emulsion with trypsin, and the other of the washed precipitate with trypsin. This last control was used to see if all the soaps could be removed from the precipitate by repeated washings with water.

Here again we have evidence that previous treatment of the emulsion with iodin increased the activity of the trypsin. The amount of incoagulable nitrogen obtained after twenty-four hours incubation of the iodized emulsion was much greater than that observed in the untreated fraction, and also greater than that obtained with the washed precipitate. It is probable that the washed precipitate was not entirely free of soaps. The amount of digestion observed after forty-eight hours in the untreated emulsion indicates that soaps were not present in large amounts. In a previous paper (9) we showed that soaps were able to inhibit completely the action of enzymes only in certain proportions, and that digestion occurred when enzymes were present in excess of this proportion. This is probably the explanation in the present instance, as the results show that soaps are present in comparatively small amounts in these pneumonic areas (text-figure 6).

In addition to the extractives of the tubercle bacilli which we have

been discussing, lipoids derived from dead cells are also present in caseous matter. Neumann (10) and others state that an alkaline solution of lecithin inhibits tryptic activity, but Meyer (11), using a 1 per cent. solution, was unable to confirm these observations.

In view of the uncertainty of the action of the lipoids we made a few experiments in order to determine their influence on enzyme activity. For these experiments lecithin, cuorin, and cholesterol were used. The solutions were prepared by mixing one gram of the lipoid with 100 cubic centimeters of a 1 per cent. solution of an inactive preparation of sodium oleate. This sodium oleate prepara-



TEXT-FIG. 6. Effect of soaps of tubercle bacilli and lipoids on tryptic digestion.

tion was one in which the acid had become saturated, thereby rendering it inactive as an inhibiting agent. By suspending the lipoids in an inactive soap solution we hoped to duplicate the physical condition present in the control containing the soap prepared from tubercle bacilli. Text-figure 6 gives the results obtained in one of the experiments.

In addition to the preparations mentioned above, there was also tested in this experiment a soap prepared by saponifying the acetone insoluble fraction of the extractives of the tubercle bacilli. The soap is apparently much less active than that prepared from the acetone-soluble fraction.

All the lipoids when tested in this manner appear to have some inhibiting action, particularly lecithin which is the most active. This experiment indicates that lipoids in the tissues, particularly in the presence of soaps, may aid in preventing autolysis, but their activity is much less than that demonstrated for the soaps.

#### DISCUSSION.

We believe that we are now in a position to understand more clearly the processes leading to caseation in tuberculosis. With the lodging of the bacilli there is death of some of the cells due to the action of the toxins, and a proliferation of the neighboring cells. Under other conditions the death of a small number of cells would be followed at once by their removal, either through the action of phagocytes or by autolysis; but here the phagocytes are absent, and the action of the enzymes is prevented by the presence of the soap of the unsaturated fatty acids derived from the tubercle bacilli. The anemia caused by the occlusion of the blood vessels plays an important part, as the lack of fluid prevents the dilution and subsequent absorption of the inhibiting substances. As the local process spreads slowly, the reaction at the periphery of the area prevents the entrance of fluids, and thus the caseous area gradually increases in size as the bacilli invade and cause the death of the surrounding tissues.

In conditions such as those present in caseous pneumonia, there is primarily an exudate composed of desquamated cells, leucocytes, and fibrin, which soon becomes caseous. Before caseation is complete in the zone of gelatinous pneumonia, there is not the anemia due to occlusion of vessels that is seen in the more chronic processes, and we must conclude that persistence of caseation is due to substances inhibiting the action of the ferment present in the inflammatory exudate. An important factor in the acute process under discussion is the immense number of bacilli present. Under these conditions

it is not difficult to imagine a corresponding increase in the amount of inhibiting substances, an increase sufficient to neutralize the ferment liberates by the disintegration of leucocytes and other cells. Caseous areas may persist for a long time without undergoing absorption. The rapid softening of these areas subsequent to secondary infections is probably due not only to the entrance of fresh ferment, but also to the fluid accompanying these conditions, the fluid acting as a diluent and aiding in the removal of the inhibiting substances. Soaps if present must exist in very minute quantities in the bacilli. They are probably formed from the neutral fat of the bacilli. The lipases hydrolyze the fats, and the acids are then saponified. Klotz (12) and Bergell (13) have demonstrated the presence of lipases in tuberculous pus and lymphocytes, while Wells and Corper (14) found the ferment present in the tubercle bacilli.

According to Sata (15), most of the fat present in caseous matter is found at the periphery of the area. This may be due either to a wandering in from the outside, or, more probably, to synthesis from the fatty acids liberated during the disintegration of the cells and of the tubercle bacilli. Our experiments indicate that soaps of the unsaturated fatty acids obtained from tubercle bacilli are the active agents in preventing autolysis, and the identification of these acids will probably aid in determining the origin of the neutral fat found in caseous areas.

In our paper on the ferment-inhibiting substances present in tubercle bacilli (16) we reported that iodin combined with the unsaturated fatty acids obtained from the bacilli and neutralized their ferment-inhibiting properties. We also emphasized the importance of this observation in connection with a specific chemotherapeutic agent for tuberculosis. In our present work we have shown that iodin exerts a similar action on the ferment-inhibiting substances present in caseous matter. Thus the iodin may serve another purpose by aiding in bringing about solution and absorption of the caseous matter, and exposing the bacilli, which otherwise are more or less inaccessible, to the influence of the therapeutic agent.

It is a well known clinical observation that iodides cause tubercle bacilli to appear in the sputum of patients with pulmonary tuberculosis, though they were previously absent. This phenomenon is

probably due to the neutralization of the action of the ferment inhibiting substances by the iodin. Subsequent to this neutralization ferment action ensues, with liberation of the bacilli and their appearance in the sputum.

The autolysis that occurs in the center of large anemic infarcts and the lack of autolysis at the periphery, can be explained in the same manner. At the periphery of the infarct the fatty acids which are liberated following the death of the cells are saponified by the alkalies present in the tissues, and by those coming from the surrounding fluids. The soaps thus formed inhibit proteolytic enzymes until the phagocytes, invading the periphery, open up channels through which sufficient fluid can enter to wash out or dilute the soaps so that autolysis can proceed. The phagocytes of course also take part in the removal of the dead tissues. In the center of the infarct the alkalies are soon used up and the tissues then become acid in reaction on account of the excess of fatty acids. Wiesner (17) found that autolytic enzymes act best in an acid medium, and this is to be expected as soaps are inactive as inhibiting agents under these conditions.

#### CONCLUSIONS.

1. Caseous matter obtained from lymph glands which have become secondarily infected contains substances which inhibit proteolytic enzyme activity. These substances consist chiefly of soaps of unsaturated fatty acids.
2. The inhibiting substances are present in relatively small amounts when the caseous matter has become secondarily infected. This is probably due to the dilution and washing out of the soaps.
3. Ferments are either entirely absent or present in very small amounts, unless the caseous matter has become secondarily infected.
4. Caseous material from the lungs contains smaller amounts of the inhibiting substances. This may be due to the acuteness of the process, which does not permit an accumulation of the soaps, or to the binding of the soaps with the ferments.
5. Ferments are present in caseous pneumonia. In the whole emulsion the ferments are less active in an alkaline than in an acid reaction; but removal of the soaps shows that those active in an alkaline reaction are also present in considerable amounts.

6. The previous treatment with iodin of caseous matter from both lymph glands and lungs increases the action of the trypsin.

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# THE EFFECT OF DILUTION OF PLASMA MEDIUM ON THE GROWTH AND FAT ACCUMULATION OF CELLS IN TISSUE CULTURES.\*

By ROBERT A. LAMBERT, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons of Columbia University, New York.)

PLATES 43 TO 45.

Since the method of cultivating tissues *in vitro* will no doubt be used in investigating the action of various substances on the growth and function of cells, it is important that the influence of cells, growing under these conditions, of certain easily modified physical and mechanical factors should be understood. The importance of these factors is indicated by the fact that so simple a matter as the thickness of the hanging drop of plasma influences markedly the extent of the outgrowth of cells from the original fragment of tissue.

In a former paper<sup>1</sup> we discussed the influence of temperature, including the effect of variations in the temperature of incubation as well as the effects of exposure of the cultures to high and low temperatures before incubation. In the present paper we shall consider the influence of another physical factor, namely dilution of the plasma medium, on the behavior of cells in cultures. Carrel and Burrows<sup>2</sup> (1911) were the first to describe the effect of dilution with distilled water on cell wandering, and the subject was also discussed in a recent paper by Burrows<sup>3</sup> (1913). Some of the work reported in this paper was begun soon after the appearance of the experiments of Carrel and Burrows, with the object of testing the correctness of the interpretation of their results regarding the

\* Received for publication, January 24, 1914.

<sup>1</sup> Lambert, R. A., *Anat. Rec.*, 1912, vi, 91.

<sup>2</sup> Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

<sup>3</sup> Burrows, M. T., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

stimulating action of hypotonic plasma. Later, the use of the method of dilution for the study of the accumulation of fat by cells *in vitro* was suggested to us, and further experiments with this aim in view were carried out. We shall report here the results of two sets of experiments, (1) upon the effect of dilution on the migration and multiplication of cells in cultures, and (2) upon the effect of dilution on the accumulation of fat by cells in cultures.

#### THE EFFECT OF DILUTION OF PLASMA ON THE MIGRATION AND MULTIPLICATION OF CELLS.

Carrel and Burrows<sup>4</sup> reported that the addition of a limited quantity of water to chick plasma caused a more extensive outwandering of cells in cultures of chick embryo spleen. They also observed that the addition of hypertonic salt solutions was harmful. The stimulating effect in the first instance was attributed to the hypotonic state of the medium. No experiments with isotonic solutions as diluents were made. Burrows has recently published the results of studies upon the effect of the addition of such solutions. A summary of his work is given below in connection with the results that we have obtained.

We have repeated the experiments of Carrel and Burrows with hypotonic plasma, preparing at the same time cultures in plasma diluted with serum and with Ringer's solution. In addition to chick embryo spleen, we have used for cultivation chick embryo heart, bone marrow and intestine, rat embryo skin, and mouse carcinoma.

*The Effect of Dilution on the Migration of Cells.*—The first experiments consisted in the preparation of cultures of chick embryo spleen in the following media: (1) undiluted plasma, (2) plasma diluted with distilled water in the proportion of 3 to 2 (the optimum medium of Carrel and Burrows), (3) plasma similarly diluted with Ringer's solution, and (4) with serum, and (5) plasma diluted with serum in the proportion of 1 to 2.

Care was taken to have the pieces of tissue of uniform size, and the drops of plasma of as nearly uniform thickness as possible. Ten preparations were made with each medium. At the end of forty-eight hours' incubation, after which time experience had

<sup>4</sup> Carrel, A., and Burrows, M. T., *loc. cit.*

shown that little further migration of spleen cells took place, cover-glasses with the clots attached were placed in formalin later mounted on slides. With a projection drawing apparatus rough drawings were made in which the pieces of tissue and halos of migrated cells were outlined. By taking the average of radii of the tissue fragments and the average width of the cell halo a composite drawing for each series of preparations was constructed. While the cultures in a single series differed little from one another the composite drawings of the several series showed certain differences (figures 1, 2, 3, 4, and 5). The most extensive migration was seen to have taken place in the plasma diluted with two volumes of serum. There is little difference in the extent of migration among the pieces of tissue in plasma diluted with the smaller quantities of serum, Ringer's solution, and water, although in each the outgrowing was more extensive than in the controls in undiluted plasma. In other words, the most active migration took place in the plasma diluted with the largest quantity of fluid, and not in the medium made hypotonic by the addition of distilled water. This result is in agreement with the recent work of Burrows in which he found that the extent of migration of spleen cells in plasma diluted with varying quantities of serum increases with the amount of serum added. The effect is attributed to the diminution in the amount of fibrin in the clots. Since this reduction in fibrin results, in the beginning at least, in a coarser meshwork in the clot, the explanation of the increased migration of cells would seem to lie in the diminished resistance to the progressive movements of the cells. In order to test this explanation several other tissues were used, including chick embryo heart, intestine and bone marrow, rat embryo skin, mouse carcinoma, the cells of which vary considerably in their migratory ability.

Briefly stated, it was found that cells which are relatively small and actively motile, such as those of the bone marrow and spleen, show increased migration in diluted plasma, whereas cells whose power of locomotion is limited, such as those of intestinal epithelium, rat embryo skin, and mouse carcinoma, which tend to spread out slowly in cultures in groups or large sheets, are not influenced by dilution of the medium. Cells which occupy a midway position

n the matter of motility, such as connective tissue cells, are only slightly influenced in this way. The mechanical explanation of diminished resistance to active cell locomotion offered by the coarser fibrin meshwork seems to us to account for these results.

*The Effect of Dilution on Cell Multiplication.*—An analysis of the results suggests other criteria for determining a true stimulating effect on tissue cultures than the extent of the outwandering of cells through the clot. It occurred to us that the extent of cell multiplication, as determined by the number of cells that undergo division in the preparations in a given time, would afford a much more satisfactory test. As we have pointed out, all stages of mitotic division can be observed with comparative ease in the living cell. Since division at body temperature is completed, as a rule, in from twenty to thirty minutes, the number of mitoses in a preparation may be hourly recorded without danger of counting the same cell twice. By making observations on small groups of preparations from the several series over a period of twenty-four hours or longer it is possible to arrive at a definite conclusion as to which series shows the most active multiplication of cells. We have studied cultures of chick embryo heart in pure plasma and in plasma diluted with water and with Ringer's solution in the proportions given above. There were five preparations in each set. Each preparation was examined six times during a period of forty-eight hours. The total number of mitoses for each of the three sets in the order stated was, 88, 96, and 81. In another experiment in which four observations were made the total numbers were, 76, 62, and 70. We concluded from these two experiments that dilution of plasma within these limits is without appreciable effect on cell multiplication.

#### THE EFFECT OF DILUTION ON THE ACCUMULATION OF FAT BY THE CELLS.

In previous communications we have referred to the appearance in the cells of tissue cultures of small droplets of fat which tend to increase in size and number with the age of the culture. After five to seven days the cells may be actually distended with the droplets.

We<sup>5</sup> reported observations which seemed to show that the accumulation was in all probability the result of a disturbance in cell metabolism. There was evidence that the vitality of the cells was not seriously impaired, since they were seen to move about actively and to undergo normal mitotic division. We also observed that cells growing in a thin drop of plasma accumulated less fat than those in a thick coagulum. This phenomenon can be easily demonstrated in a single preparation by shaking the drop of plasma before coagulation takes place, causing the drop to touch the slide cavity, and then by manipulating the tissue fragment so that it lies along the inner edge of the drop. The cells that grow in one direction into the thick clot accumulate a large quantity of fat in their cytoplasm, while those growing in the other direction in the thin film of medium on the cover-glass accumulate very little fat. It is difficult to say whether this difference in fat accumulation is due to the difference in the quantity of fat at the disposal of the cells, or to a difference in oxygen supply to the cells under the two conditions, as has been suggested by Burrows.

Several methods suggested themselves for testing the effect of a reduction in the quantity of fat in the medium on the amount of fat accumulated by the cells: (1) the removal of the fat by extraction with ether or chloroform; (2) the use of an artificial fat-free medium, such as agar or salt solutions, in which Lewis<sup>6</sup> states that embryonic chick cells will grow for a time; and (3) the use of plasma in which the amount of fat has been reduced by dilution with Ringer's or physiological salt solution. The first method was found impracticable because prolonged shaking of serum with pure ether or chloroform seemed to produce some change, probably in the serum proteins, as the result of which the serum even when combined with sufficient plasma to cause clotting did not afford a satisfactory culture medium. The second method could not be successfully used, because, after washing the tissue fragments in salt solution to remove the tissue lymph before preparing the cultures, it was found that the cells in the outgrowths lived only eighteen to twenty-

<sup>5</sup> Lambert, R. A., and Hanes, F. M., *Virchows Arch. f. path. Anat.*, 1913, ccxi, 100.

<sup>6</sup> Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

four hours; that is, not long enough to make comparative observations. Very little fat is present in the pure plasma preparations at the end of twenty-four hours' incubation. We therefore resorted to the use of the third, or dilution method. Dilutions of chick plasma in Ringer's solution were made in the following proportions: 1 part of plasma to 2, 5, 10, 12, 15, and 20 parts of Ringer's solution. Clotting was not interfered with by the high dilution of the plasma, although the coagula formed by drops of the 1 to 15 and 1 to 20 dilutions were often not firm.

It was found that cells from pieces of chick embryo heart, previously washed in salt solution, showed active migration and multiplication in each of the diluted media. However, those growing in the 1 to 20 medium underwent early disintegration, frequently in less than twenty hours. The lower dilutions only were therefore used in the majority of the experiments. A single protocol will be sufficient to show the effect of dilution on the accumulation of fat by the cells; for it was found that when proper care was exercised in the preparation of the cultures, including the thorough washing of the pieces of tissue to prevent a relatively great modification of the dilution from the addition of tissue lymph, and in the care of the instruments and glassware used, the results in a number of experiments were practically uniform.

*Experiment iii-5.*—Plasma was obtained by bleeding a fowl from the wing vein. The heart of a ten day chick embryo was cut into small pieces of the size suitable for culture preparations. Dilutions of plasma were made by adding 1 part to 2, 5, 10, 12, and 15 parts of Ringer's solution. Ten pieces of tissue were put up in each medium, including undiluted plasma.

All preparations upon examination after twenty-four hours showed the usual radial extension of spindle and irregularly shaped cells. Small droplets of fat were visible in the cells in pure plasma and in those in the 1:2 and 1:5 dilutions; only occasional granules were seen in the preparations of the remaining series. After forty-eight hours the differences were more striking. There was a marked increase in the number and size of the granules in the cells in pure plasma and in the 1:2 dilution. In the 1:5 dilution the cells showed a moderate amount of fat, but decidedly less than those in undiluted plasma. Those growing in the higher dilutions were practically free from fat. Three of the preparations from each series were fixed in formalin at this time in order that the observations on the living cells could be verified by staining with Sudan III (figures 6 and 7). Some of the preparations in diluted media became disintegrated on the third and fourth days. Those remaining alive as long as four days presented striking pictures when compared with the controls, the former

showing only occasional granules of fat, while in the latter the cells appear distended with numerous large droplets.

In order to exclude the possibility that in the diluted plasma the diminution in the amount of fibrin might have influenced in some way the fat metabolism of the cells, plasma diluted with serum instead of salt solution was used in another set of experiments. It was found that the accumulation of fat under these circumstances was practically the same as in undiluted plasma. Repetitions of these experiments, including several in which pigeon spleen and mouse carcinoma were used, gave results that varied little from those just stated. Two further observations, however, should be mentioned. First, it was occasionally observed that some of the cells that wandered farthest into the clot of highly diluted plasma showed a small number of rather large fat droplets, while the cells in the denser outgrowth immediately about the tissue fragment were practically fat-free; secondly, in a few of the preparations in diluted plasma in which the outgrowth consisted of only fifteen to twenty cells, these cells contained more fat than those in preparations showing a luxuriant outgrowth. These observations seem to be in harmony with the findings stated, for it may be assumed that if the number of cells in a culture or in a particular area of the clot is small the fat supply for these cells is relatively greater.

It has therefore been concluded that the effect of dilution of plasma with salt solutions reduces the quantity of fat accumulated by the cells by reducing the quantity of fat in the medium.

#### SUMMARY.

1. Dilution of plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produces the same effect. Less active motile cells are influenced little or not at all by dilution. The effect on cells of the first type is probably due to the reduction in the quantity of fibrin in the clot producing lessened resistance to cell locomotion.

2. Dilution of plasma with either isotonic solutions or distilled water is without effect on cell multiplication, as is shown by records of the number of mitoses in living culture preparations.

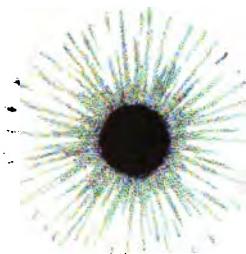


FIG.1.

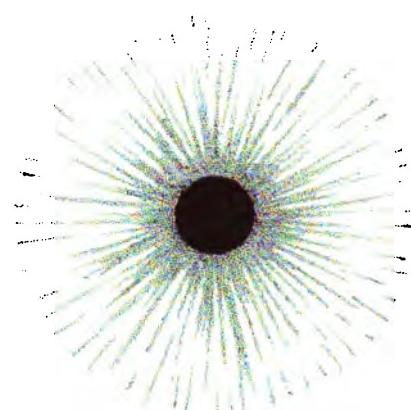


FIG.2.

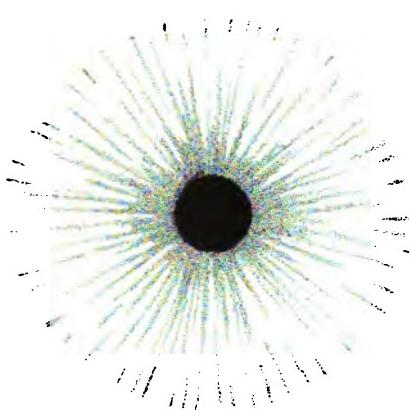


FIG.3.

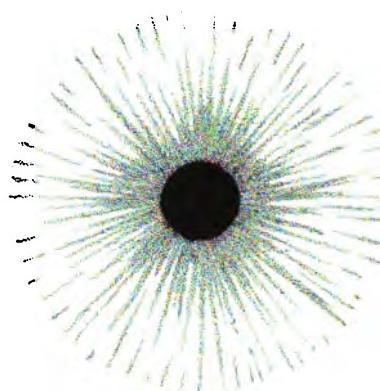


FIG.4.

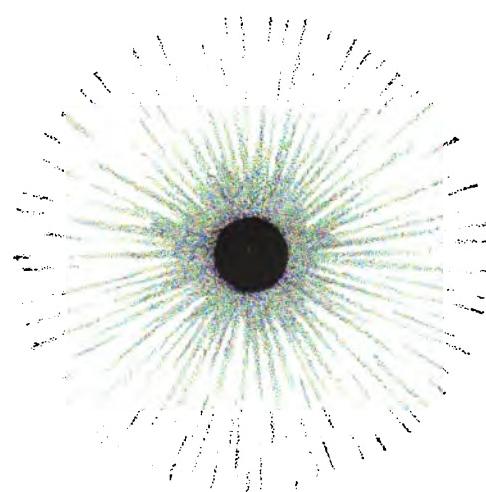


FIG.5.

(Lambert: Growth of Cells in Tissue Cultures.)



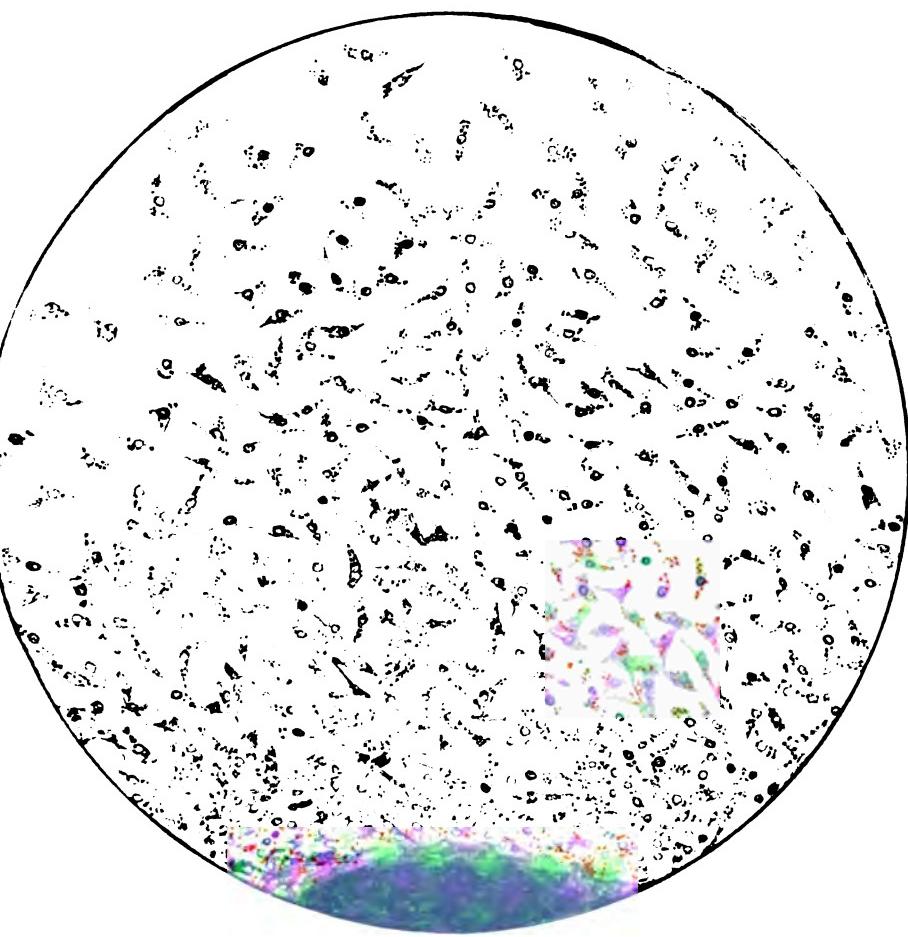


FIG. 6.

(Lambert: Growth of Cells in Tissue Cultures.)



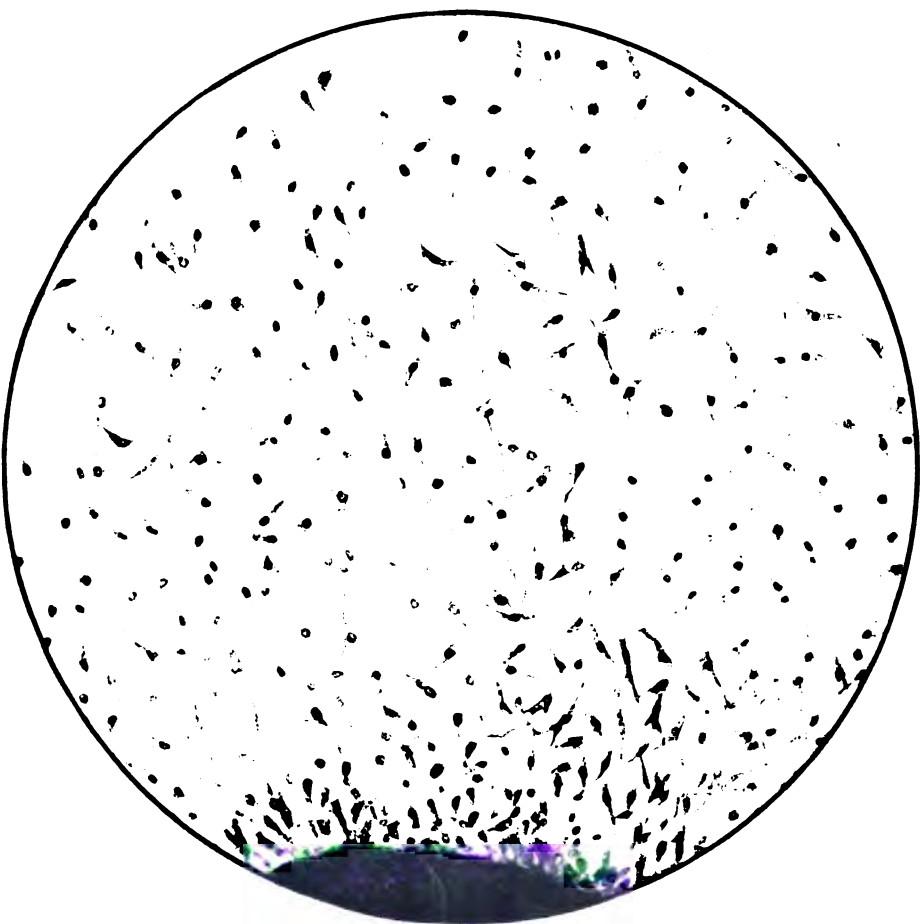


FIG. 7.

(Lambert: Growth of Cells in Tissue Cultures.)



3. Dilution of plasma with suitable quantities of Ringer's solution causes a marked diminution in the quantity of fat accumulated by the cells. This reduction is to be attributed to the decrease in the quantity of fat in the medium. The accumulation of fat by cells in cultures is therefore not to be regarded as the result of a cell degeneration, but as an accumulation, the source of the fat being the medium in which the cells are growing.

#### EXPLANATION OF PLATES.

##### PLATE 43.

Drawings showing the extent of migration of spleen cells in undiluted chick plasma and in plasma diluted with serum and with salt solutions.

FIG. 1. In undiluted chick plasma (control).

FIG. 2. In plasma diluted with serum in the proportion of 3:2.

FIG. 3. In plasma diluted with distilled water in the same proportion.

FIG. 4. In plasma diluted with Ringer's solution in the same proportion.

FIG. 5. In plasma diluted with serum in the proportion of 1:2.

##### PLATE 44.

FIG. 6. Three day culture of chick embryo heart in undiluted plasma. Stained with hematoxylin and Sudan III to show the accumulation of fat by the cells.

##### PLATE 45.

FIG. 7. Three day culture of chick embryo heart in plasma diluted with Ringer's solution in the proportion of 1:15. The staining shows that fat granules are practically absent. It is also to be observed that the cells are smaller and stain somewhat more deeply with hematoxylin than in the control preparation.

## A NOTE ON THE PATHOGENICITY OF TRYPANOSOMA LEWISI.\*

By WADE H. BROWN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

In the usual scheme of classification of trypanosomes, *Trypanosoma lewisi* occupies the position of the type species of a number of non-pathogenic trypanosomes.<sup>1</sup> While this usage is justifiable in the present state of our knowledge of these organisms, one must not lose sight of the fact that there is abundant evidence to show that *Trypanosoma lewisi* is not strictly non-pathogenic, but occasionally manifests a decided virulence for rats, especially young ones. Apart from such frequent disturbances as fever, anemia, and loss of weight, a considerable mortality may occur among infected rats. Perhaps the best instance that can be cited is that reported by Jürgens<sup>2</sup> who noted a mortality of 29.3 per cent. (16 out of 47) among young rats. Other authors have noted a slight mortality or no mortality, resulting from infections of *Trypanosoma lewisi*. These differences in pathogenicity indicate that there are strains that differ fundamentally as regards their virulence. Delanoë<sup>3</sup> has added support to this conception of pathogenic and non-pathogenic strains of *Trypanosoma lewisi* by showing that while certain strains, or organisms from certain sources, are incapable of infecting mice, other strains may infect even a considerable percentage (40 per cent.) of the mice inoculated. Further, Roudsky<sup>4</sup> has shown by his "reinforced virus" that the virulence of a given strain is not absolutely fixed, but that it can be markedly increased for both rats and mice. Finally, Wendelstadt and Fellmer<sup>5</sup> have succeeded in

\* Received for publication, February 15, 1914.

<sup>1</sup> Laveran, C. L. A., and Mesnil, F., *Trypanosomes et trypanosomiases*, 2nd edition, Paris, 1912, 241.

<sup>2</sup> Jürgens, *Arch. f. Hyg.*, 1902, xlvi, 265.

<sup>3</sup> Delanoë, P., *Compt. rend. Soc. de biol.*, 1911, lxx, 649.

<sup>4</sup> Roudsky, D., *Compt. rend. Soc. de biol.*, 1910, lxviii, 421, 458; 1911, lxx, 74.

<sup>5</sup> Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 422; 1910, v, 337.

raising the virulence of *Trypanosoma lewisi* by passage through cold-blooded animals.

These facts suffice to show the existence of pathogenic strains of *Trypanosoma lewisi* and indicate that possibly all strains possess potential pathogenic properties. Unfortunately, the known facts regarding its pathogenicity are too meagre to warrant any generalizations. Since the natural host of this organism makes it peculiarly suited to laboratory study, further work upon this subject seems highly desirable, as a clearer comprehension of the conditions governing its pathogenicity would probably aid materially in advancing our knowledge of the more important group of organisms, —the pathogenic trypanosomes.

Recently, we have had under observation a strain of *Trypanosoma lewisi* that, for a short time, showed an unusual increase in its virulence, and an account of the action of this organism is offered as a contribution to the study of its pathogenicity.

A complete genealogy of this strain of *Trypanosoma lewisi* is not available. The organism was isolated from a natural infection in a wild rat and has been carried for several years in white rats with no unusual manifestations of virulence. In October, 1913, for three successive generations, fatal infections resulted from the intraperitoneal injection of one to two drops of tail blood, diluted with one cubic centimeter of a 1 per cent. sodium citrate solution, death taking place eight to eleven days after inoculation. Five rats out of the six inoculated succumbed to this infection. At first it appeared improbable that *Trypanosoma lewisi* was entirely responsible for such an unusually high mortality, but as careful investigation, including cultures from the peritoneal cavity and heart's blood, failed to reveal any other cause, the following test of virulence was applied.

*Experiment 1.*—Nov. 28, 1913. A large rat infected with strain I of *Trypanosoma lewisi* was bled to death on the twelfth day of infection and the blood defibrinated. At this time there was a heavy blood infection with a few multiplication forms still present. The rat was weak and showed a marked anemia and loss of weight. With aseptic precautions, ten normal rats were injected intraperitoneally with 0.2 c.c. of blood diluted with 0.8 c.c. of an 0.85 per cent. salt solution. Five of the rats weighed between 100 and 170 gm., and the other five between 40 and 70 gm. As a control, four normal rats (two large and two small) of the same lot were kept under observation and two large immune rats were injected intraperitoneally with 0.5 c.c. of blood in 1 c.c. of salt solution.

Nine of the normal rats inoculated showed trypanosomes in the blood within twenty-four hours. The tenth rat, a large one, showed a very few organisms at forty-eight hours, but the infection was transient, disappearing completely within five days without any demonstrable multiplication having taken place; this rat was probably an immune. Another large rat died in forty-eight hours and at the autopsy showed a marked bronchiectasis. If we exclude these two rats from the list of normal ones, the eight remaining rats showed a rapid increase in trypanosomes in the blood with definite multiplication on the third day, persisting until the fatal termination of the infection. The rats became torpid and weak, with marked anemia and dyspnea, and slight loss of weight. A few rats showed bloody nasal and lachrymal discharges, and all of them developed diarrhea with greater or less abdominal distension. Two of the large rats died on the sixth day, and the third died on the seventh day, while all five of the small rats died on the ninth day after inoculation.

Autopsies on these rats showed an acute enteritis, marked splenic enlargement and hyperplasia of the bone marrow, and a few foci of necrosis in the liver. Several rats showed a moderate pulmonary congestion but no bronchiectasis or pneumonia. Cultures made immediately after death from the peritoneal cavity and from the heart's blood of three rats remained sterile. No cultures were taken from the other rats.

The normal and immune controls remained unaffected for one month, when observations were discontinued.

The existence of nasal and lachrymal discharges and diarrhea in these rats would naturally incline one to suspect some condition complicating the trypanosomiasis. In an attempt to clear up the relation of these conditions to the trypanosomal infection, a number of observations have been made on normal and immune rats inoculated with different strains of *Trypanosoma lewisi*. These observations show that the above conditions may recur frequently in a given series of inoculations while they do not appear in control immunes inoculated from the same source, in uninoculated normal rats from the same lot, or in rats inoculated with another strain of *Trypanosoma lewisi*. Likewise, normal and immune rats kept in

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contact with infected rats showing these conditions, with a single exception, have not developed these symptoms except as the normal rats became infected with trypanosomes by natural means. The fact that the same symptoms occur in the course of other trypanosomal infections in the rat indicates that they may be a part of the morbid manifestations of trypanosomiasis. Whether these conditions are the result of an uncomplicated trypanosomal infection or the result of a latent infection whose development is favored by the trypanosomiasis is not clear. In either case, however, it is evident that it is the trypanosome that is directly or indirectly responsible for the condition.

The series of fatal infections in the stock transfers of this organism was interrupted by inoculating two young rats weighing seventy grams, with one drop of tail blood, taken on the tenth day of infection, from the rat killed for the above experiment. One of the rats was extremely ill for about twenty days but finally recovered. The infection in the other rat was much less severe. On the next transfer from the first of these rats, one large and one small rat were inoculated and the resulting infections were still further decreased in severity.

The small rat of this series was then sacrificed for a second test of the virulence of this strain of *Trypanosoma lewisi*, in this instance contrasted with another strain, recently isolated from a natural infection in a white rat.

*Experiment 2.*—Dec. 17, 1913. A young rat infected with strain I of *Trypanosoma lewisi* and another young rat infected with strain V were bled from the heart on the eighth day of infection and the blood was collected and defibrinated under aseptic conditions. The blood of the two rats showed about an equal number of trypanosomes with many multiplication forms. From each of these rats, five rats weighing 80 to 90 gm. were inoculated intraperitoneally with 0.2 c.c. of blood diluted with 0.8 c.c. of salt solution.

The five rats infected with strain I all showed an incubation period of less than twenty-four hours. Three of the five rats developed a severe infection with weakness, a high grade anemia, and loss of weight. The other two showed only a moderate infection. None of the rats died and trypanosomes were present in the blood of four on the thirtieth day, when observations were discontinued. Of the five rats infected with strain V, the incubation period of four was

less than twenty-four hours, of the fifth between thirty-six and forty-eight hours. Only one of these rats showed any appreciable disturbance from the infection. This rat showed an extreme blood infection, with weakness, torpor, anemia, and loss of weight, persisting for about three weeks. Trypanosomes disappeared from the blood of two of these rats within twenty days and from a third by the twenty-sixth day.

Although none of the ten rats died from the infection, the difference in the severity of the infection produced by the two strains of *Trypanosoma lewisi* was sufficiently well marked to show that strain I was decidedly more virulent than strain V.

This series of observations lends support to the idea that there are strains of *Trypanosoma lewisi* that differ fundamentally as to their pathogenicity, but what is of even greater importance is that any particular strain is subject to marked fluctuations of virulence. From these facts it would seem important to determine the conditions that give rise to such alterations of virulence as have been described, the extent to which these variations of virulence more or less permanently modify the characteristics of the strain, and, finally, whether there are differences in morphology corresponding with variations in virulence or with the differences in pathogenicity exhibited by different strains of *Trypanosoma lewisi*. These subjects are now under investigation.

#### CONCLUSIONS.

1. Some strains of *Trypanosoma lewisi* may, at times, produce rapidly fatal infections in a large percentage of the rats infected.
2. In such strains of *Trypanosoma lewisi*, a sufficient degree of pathogenicity may persist to warrant the designation of these strains as pathogenic.
3. The pathogenicity of a given strain of *Trypanosoma lewisi* is not constant, but is subject to marked and even sudden variations.

## PENETRATION OF THE VIRUS OF POLIOMYELITIS FROM THE BLOOD INTO THE CEREBRO- SPINAL FLUID.\*

BY SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

An active poliomyelic virus readily causes infection in monkeys when introduced into the brain, subarachnoid spaces, or peripheral nerves. When, however, the virus is injected into the circulation, far greater quantities are required to produce infection and the onset of the usual symptoms and the paralysis are delayed.<sup>1</sup> Were the virus withdrawn from the blood directly by the tissues of the central nervous system neither the greater dose nor the longer incubation should be necessary. It appears that the extranervous organs retain but little of the virus; hence we may suppose that the route by which the virus contained within the blood reaches the central nervous system is an indirect one.

It is now generally conceded that the poliomyelic virus enters the human body by way of the upper respiratory passages, and in particular through the nasopharyngeal mucous membrane. Once within this membrane the virus may pass through the lymphatic channels surrounding the filaments of the olfactory nerve to the leptomeninges where it reaches the cerebrospinal fluid, or it may first enter the blood and be conducted to the central nervous organs by the general circulation. Flexner and Clark<sup>2</sup> have shown experimentally that when the virus is introduced into the upper nasal mucosa in monkeys its propagation can be followed from the olfactory lobes of the brain to the medulla oblongata and spinal cord. Had the distribution of the virus taken place by way of the general circulation the several parts of the nervous organs should have been

\* Received for publication, February 26, 1914.

<sup>1</sup> Clark, P. F., Fraser, F. R., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 223.

<sup>2</sup> Flexner, S., and Clark, P. F., *Proc. Soc. Exper. Biol. and Med.*, 1912-13, x, 1.

rendered infectious almost simultaneously. The virus of poliomyelitis has hitherto been regarded as strongly neurotropic; but it does not follow from the fact of this neurotropic affinity that the nervous tissues can under all circumstances remove the virus from the blood.

Another possibility exists, namely, that the virus introduced into the blood finds its way not directly to the nervous organs, but indirectly by way of the cerebrospinal fluid. When, therefore, a quantity introduced into the blood is insufficient to cause infection, although a much smaller quantity produces infection when introduced into the brain, the reason may be that the intact choroid plexus prevents the virus from reaching the cerebrospinal liquid. It is well known that this anatomical barrier excludes from the cerebrospinal fluid many substances contained within the blood, that the barrier is not absolute but is capable of being broken down, and that the most frequent source of injury is the pathogenic action of infectious microorganisms.

Hence we may consider that when insufficient quantities of the poliomyelic virus are introduced into the blood they do not set up poliomyelitis because they fail to injure the choroid plexus; and when poliomyelitis is set up by larger quantities the plexus has been penetrated.

The virus has thus far not been detected by inoculation experiments in the cerebrospinal fluid obtained from human cases of poliomyelitis; and, indeed, when the virus is injected into the subarachnoid spaces in monkeys it remains within the fluid for a limited time and can no longer be detected there at the period of the onset of paralysis.<sup>8</sup> The conditions within the fluid are obviously unfavorable for the propagation of the virus; but the fluid constitutes the most immediate route for the passage of the virus to the interior of the nervous organs in which it multiplies and becomes fixed. The cerebrospinal fluid therefore acts merely as a medium for transporting the virus to the nervous tissues. Hence, should the passage of the virus contained within the blood proceed to the nervous organs by way of the cerebrospinal fluid it should be possible to detect it in transit. From the many failures to discover the virus in

<sup>8</sup> Clark, Fraser, and Amoss, *loc. cit.*

the early paralytic stages of poliomyelitis in man and the monkey it might appear that the probability of detecting the virus at all might be small. But it would now appear that when a large dose of the virus has been injected into the blood and a sufficient period allowed to elapse in order that the virus may act upon and injure the choroid plexus, it is possible to detect the presence of the virus in the cerebrospinal fluid by inoculation.

#### EXPERIMENTAL.

A passage strain of highly active K virus was employed for the intravenous inoculation. A Berkefeld filtrate of a 5 per cent. suspension caused paralysis and death when inoculated intracerebrally in doses of 0.2 to 0.3 of a cubic centimeter. For the purpose of intravenous inoculation the 5 per cent. suspension was merely centrifugalized and the clear supernatant fluid pipetted off. It was not filtered.

*Experiment A. Macacus rhesus 1.*—Jan. 30. 250 c.c. of the supernatant fluid were injected into a superficial vein of the leg. No effect was produced by the injection. No symptoms appeared until Feb. 14, when slight excitability was noted. Feb. 16. Weakness of the leg. Feb. 17. Excitability increased and arms paralyzed. Feb. 18. Legs and back paralyzed. Feb. 19. Died.

Lumbar puncture was performed as follows: on Feb. 1 (48 hours after the injection) 0.6 c.c. of cerebrospinal fluid was removed; on Feb. 2 (72 hours after the injection) 0.5 c.c. of fluid removed; on Feb. 3 (96 hours after the injection) 0.9 c.c. of fluid removed; and on Feb. 18 (19 days after the injection) 2.9 c.c. of fluid removed. The several samples of cerebrospinal fluid were free from blood. On Feb. 18 a sample of blood was also taken and defibrinated.

The specimens of cerebrospinal fluid and blood were injected intracerebrally into monkeys as follows:

*Macacus rhesus 2.*—Feb. 1. 0.6 c.c. of cerebrospinal fluid withdrawn from monkey 1 48 hours after an intravenous injection of K virus was inoculated intracerebrally. No symptoms were produced; the monkey remained well.

*Macacus rhesus 3.*—Feb. 2. 0.5 c.c. of cerebrospinal fluid withdrawn from monkey 1 72 hours after an intravenous injection of K virus was inoculated intracerebrally. Feb. 14. Monkey somewhat excitable, movements slow. The condition remained stationary until Feb. 25, when the excitability was more marked. No weakness of muscles was detected. Lumbar puncture yielded a clear fluid devoid of globulin and containing forty white corpuscles per c.mm. Feb. 26. Condition not so good; ataxia; slight weakness of both arms. Mar. 10. Condition stationary.

*Macacus rhesus 4.*—Feb. 3. 0.9 c.c. of cerebrospinal fluid withdrawn from

monkey 1 96 hours after an intravenous injection of K virus was inoculated intracerebrally. Feb. 7. Excitability. The condition did not change materially until Feb. 17 when excitability was greater. The condition again became stationary until Feb. 26, when a general paralysis of the muscles was present. Animal etherized. Lesions of poliomyelitis present.

*Macacus rhesus* 5.—Feb. 18. 2.9 c.c. of cerebrospinal fluid withdrawn from monkey 1 19 days after an intravenous injection of K virus and the 1st day of paralysis were inoculated intracerebrally. Feb. 26, A. M. Excitable; tremor; ataxic. P. M. Left arm flaccid. Feb. 27. Animal prostrate; all members were paralyzed except the tail and a few neck muscles. Etherized. Lesions of poliomyelitis present.

*Macacus rhesus* 6.—Feb. 18. 5 c.c. of defibrinated blood withdrawn from monkey 1 19 days after an intravenous injection of K virus and the 1st day of paralysis were inoculated intracerebrally. No symptoms developed; the animal remained well.

The experiments bring out several facts. (1) Even a very large dose of an active poliomyelic virus when injected into the blood produces a much delayed infection. If we consider the certain effective dose of the specimen of K virus employed when injected intracerebrally at 0.2 of a cubic centimeter, then 1,250 doses were introduced into the blood of monkey 1. The average incubation period after an intracerebral inoculation is about 6 days;<sup>4</sup> in monkey 1 the period was 17 days. (2) The cerebrospinal fluid removed 48 and 72 hours, respectively, after the intravenous injection failed, when inoculated intracerebrally, to communicate definite poliomyelitis to rhesus monkeys; while the fluid removed 96 hours (monkey 4) and 19 days (monkey 5) after the intravenous injection caused typical poliomyelitis in monkeys of this species. In monkey 4 the incubation period was indefinite and in monkey 5 it was 8 days. (3) By the 19th day following the intravenous injection of the large dose of the virus and at the onset of the paralysis the virus had disappeared from the blood while it was still detectable in the cerebrospinal fluid by inoculation.

#### DISCUSSION.

The experiments emphasize in the first place the relatively great difficulty of infecting monkeys with the virus of poliomyelitis by introducing it directly into the blood. At first sight it may appear that this statement is in conflict with the effects of subcutaneous or

<sup>4</sup> Clark, Fraser, and Amoss, *loc. cit.*

even of intraperitoneal inoculations. It is, however, not improbable that in all external modes of inoculation practiced, except the intravenous mode, the virus actually penetrates to the central nervous organs by way of the nerves. In any event the difficulties in the way of accomplishing infection through the general blood provide another argument against the notion that the virus of epidemic poliomyelitis is communicated to man by the bite of infected blood-sucking insects.

On the other hand, the experiments afford valuable support to the hypothesis that infection of the nervous organs in man occurs through the mediation of the cerebrospinal fluid. The virus readily traverses the nasal mucous membrane to reach this fluid, which is capable of carrying the virus to the interstices of the nervous tissues. Apparently the virus enters the intimate structures of the nervous tissues not directly from the blood but indirectly after being passed from the blood to the cerebrospinal fluid. To accomplish this transfer time is required since the barrier of the choroid plexus must first be overcome. At the expiration of 48 hours following the intravenous inoculation the barrier appears still to be intact; at the expiration of 72 hours the passage of the virus seems to have begun, since infection of mild type followed the inoculation of the cerebrospinal fluid removed at this period. At the expiration of 96 hours it appears that the barrier had broken down; and it also appears that under the pathological conditions created the virus, in quantity sufficing to cause infection, still persisted in this fluid as late as the 19th day, although no longer detectable in the blood by the inoculation test. In no other instance has the virus been found in the cerebrospinal fluid at the period of the onset of paralysis.

When we consider the minute size of the microorganism constituting the virus of poliomyelitis we may well wonder at the failure to penetrate the capillaries to gain access to the interstices of the central nervous organs. The case is not wholly unique. von Behring discovered that the hen, which is insusceptible to the action of tetanus toxin injected into the blood, is subject to its effects when introduced into the cerebrospinal fluid. Lesions of the lepto-meninges of an interstitial character are implicated in the develop-

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ment of the specific lesions of poliomyelitis. They also suffice to produce at times in man and the monkey a poliomyelic affection of the meninges in which the central nervous organs proper do not share specifically. Hence the meninges and the cerebrospinal fluid play a highly important and even a determining part in the pathogenesis of epidemic poliomyelitis.

#### CONCLUSIONS.

The virus of poliomyelitis introduced into the blood may pass indirectly by way of the cerebrospinal fluid to the interstices of the central nervous organs.

To reach the cerebrospinal fluid the virus must first penetrate the barrier of the choroid plexus, which operation requires time. By the inoculation test, no virus was detected in the fluid at the expiration of 48 hours, only small amounts at the expiration of 72 hours, while at the expiration of 96 hours the virus had passed more freely. The virus was still detectable in the fluid at the onset of paralysis 19 days after the intravenous injection.

Pathological conditions of the leptomeninges and the cerebrospinal fluid play an important part in the pathogenesis of epidemic poliomyelitis.

ST. JOSEPH'S  
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СОВЕТСКАЯ ГОСУДАРСТВЕННАЯ  
БИБЛИОТЕКА

## ON THE SIGNIFICANCE OF THE SUBMILARY MYOCARDIAL NODULES OF ASCHOFF IN RHEUMATIC FEVER.\*

By WILLIAM THALHIMER, M.D., AND M. A. ROTHSCHILD, M.D.

(From the Pathological Laboratory of the Mount Sinai Hospital, New York.)

PLATES 46 TO 48.

Aschoff, in 1904, described what he considered a specific microscopic lesion in the myocardium of cases of acute rheumatic fever. Previously Romberg and his pupils had noted that interstitial changes and inflammatory foci occur in the myocardium of cases succumbing either to the acute fever or to chronic valvular disease of rheumatic origin. Aschoff called the focal lesions "submiliary nodules of rheumatic fever," and stated that they occur near the small or medium sized vessels, to the adventitia of which they often have close relation. The lesions consist of large cells with one or more large polymorphous nuclei, the cells being arranged in the form of a fan or rosette. The central portion of the nodule is composed of faintly staining, apparently necrotic masses of protoplasm. The giant cells, so called, resemble the large cells of certain sarcomatous or pseudoleukemic growths. The nodules include also large and small lymphocytes and polymorphonuclear leucocytes. The small lymphocytes are numerous at the periphery. Aschoff believes that the giant cells arise as a result of swelling from the adventitial "*Wanderzellen*." These cells give the cell masses their characteristic appearance.

Geipel studied seven cases of rheumatic myocarditis, of which one was complicated by staphylococcus abscesses in the myocardium, and another by gonococcus endocarditis. The nodules occurred in the intermuscular connective tissue, especially about the blood vessels. He describes their origin as follows: "There is first an increase in the size of the connective tissue cells, both the nuclei and the cytoplasm taking part in the process. Later the nuclei subdivide and in some cases the cells become confluent. As a result, giant cells, 15 to 23 microns in size, develop, and these assume a peripheral arrangement about the connective tissue fibers, the latter staining faintly in the center of the nodules." Geipel's view of the origin of the nodules, namely from the interstitial connective tissue, is in contradistinction to Aschoff's view of their origin from the adventitial "*Wanderzellen*."

Geipel also made a study of the blood vessels in other organs and failed to find any lesions resembling the Aschoff bodies. He believes that the rheumatic virus exerts a characteristic effect upon the cardiac interstitial tissue, that the

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latter undergoes a certain degree of necrosis, followed by a form of productive inflammation which exhibits itself in the formation of the Aschoff bodies. He does not admit, however, that the Aschoff bodies are specific for rheumatic myocarditis, for he claims to have observed similar nodules in the heart of a case of chronic interstitial nephritis. But as adherent pericardium coexisted, there is the possibility, as suggested by Aschoff, of a previous rheumatic infection.

Coombs demonstrated the bodies in several series of cases, chiefly in the wall of the left ventricle. They were absent from non-rheumatic cases. Wächter reported a case in which the bodies were abundant, and Bracht and Wächter later found the nodules in three cases of acute articular rheumatism. However, in a fourth case, giving a rheumatic history, the bodies were absent. In two of the cases a *Streptococcus mitis*<sup>1</sup> type of organism was cultivated from the heart's blood post mortem. They failed to reproduce the lesions in animals. Saigo observed the bodies in five hearts from persons giving a previous history of rheumatism. In one instance, a woman, dying of sarcoma of the uterus, who had suffered from acute articular rheumatism twenty-seven years previously, scars with an occasional giant cell were present, a finding which he considered sufficient proof of a previous rheumatic infection. Takayasu and Löw each found the nodules in one case. Fraenkel, in order to test the specificity of the Aschoff bodies, made post-mortem studies of twenty cases in which blood cultures had been made *intra vitam*. He demonstrated the value of the Unna-Pappenheim methyl-green pyronin stain for differentiating them from other foci. Eight of his twenty cases had given a rheumatic history. In seven cases no history was available and five subjects had not suffered from rheumatism. One instance was similar to one of ours, there being a superadded vegetative endocarditis due to an infection by *Streptococcus mitis*. Of the eight cases in which a definite history of rheumatism was obtainable, five showed Aschoff bodies. In the remaining three cases the attack of rheumatism occurred three, seven, and twenty years respectively before death. However, fibrous scars occurred with a few characteristic cells in a case eighteen years after an attack of acute rheumatic fever. He calls attention to the occurrence in old cases of fibrous scars which infiltrate the myocardium. In the five cases in which there was no history of rheumatism, the nodules were absent in two, characteristic cells were found in two, and the cells and nodules in one. The fifth case was one of chorea without joint manifestations, and the characteristic nodules were present. In seven cases of verrucous endocarditis, without history, Aschoff bodies or their remains were present in six. Fraenkel believes that even in the absence of a history of rheumatism the finding of Aschoff bodies is strong presumptive evidence of a preceding rheumatic infection. In the hearts of patients dying from infection of the endocardium by the streptococcus, staphylococcus, pneumococcus, *Bacillus aerogenes*, or the diphtheria bacillus, Aschoff bodies could not be demonstrated.

Douglas found the bodies in one case of acute rheumatic fever, and Huzella found them in six cases of acute rheumatic fever, and in one case of chorea. Using the Unna-Pappenheim or other method of staining, the various authors in general confirm Aschoff's statement as to the appearance and composition of the

<sup>1</sup> This term is synonymous with *Streptococcus mitior seu viridans* (Schottmüller), *Streptococcus viridans*, *Streptococcus salivarius*, and *Streptococcus fecalis*.

foci and their relation to the blood vessels, but some share the opinion of Geipel, that the giant cells arise from the fixed cells of the adventitia.

#### MATERIAL.

The material studied consists of the following: three cases of acute rheumatic fever; three cases of chorea without rheumatic history; one case of combined verrucous and subacute bacterial endocarditis without rheumatic history; fourteen cases of subacute bacterial endocarditis in the active stage of the disease; five cases of subacute bacterial endocarditis in the bacteria-free stage; five cases of chronic valvular disease without rheumatic history; five cases of acute streptococcus endocarditis; one case of pneumococcus; three of gonococcus; and three of staphylococcus endocarditis.

#### CASES SHOWING ASCHOFF BODIES.

Cases 1, 2, and 3 of our series were cases of typical acute articular rheumatism. Blood cultures, made *intra vitam*, in case 1 were negative; in cases 2 and 3 a search for microorganisms in the heart was unsuccessful. Case 1 had three attacks of acute articular rheumatism and finally developed an acute pericarditis. Careful study of the heart showed that each attack had left its imprint in the myocardium. We found scars with an occasional giant cell of the Aschoff type,—a healed or healing lesion. A more recent stage was also present, showing an intermingling of Aschoff cells and fibroblasts. In addition very recent bodies were found in the myocardium, probably depending upon the last rheumatic manifestation, namely, pericarditis.

Case 2 shows the most extensive involvement of the myocardium in our series. Several Aschoff bodies are found in each microscopical field. Case 4 corresponds to a case which Fraenkel reported (case 9) in which there was no definite history of rheumatism. Blood cultures during life showed *Streptococcus mitis*; however, post-mortem examination showed both verrucous and vegetative endocarditis. The mitral valve and myocardium showed numerous bodies in the stage of fibrosis; besides this, lesions identical with those produced experimentally by Bracht and Wächter are present.<sup>2</sup> We would interpret this as a rheumatic process with an

<sup>2</sup> Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 429.

added bacterial involvement, as previously damaged valves are prone to secondary infections. The fact that the bodies were not present in any of the fourteen cases of subacute bacterial endocarditis reported in this paper leads us to agree with Fraenkel that even in the absence of a history of rheumatism, the finding of the bodies is strong presumptive evidence of a previous rheumatic infection.

Cases 5, 6, and 7 had a history of chorea and are of interest because the pathological lesion proves the theory which clinicians have long held, that rheumatism and chorea are closely related. None of the cases had any articular manifestations. The myocardium in case 5 showed numerous bodies with a considerable fibrosis and in addition a diffuse infiltration with Aschoff cells. The sudden cardiac death in this case might possibly be explained by the diffuse invasion of the myocardium. Cases 6 and 7 showed only a few small bodies. In none of these cases have we been able to demonstrate bacteria in the lesions with either the Gram-Weigert, cresyl-violet, or Unna-Pappenheim stains.

*Case 1.*<sup>3</sup>—C. R., age 17 years, had been a patient in Mount Sinai Hospital three times previously with acute articular rheumatism and a valvular defect. The patient returned to the hospital with dyspnea, palpitation, and fever.

*Autopsy. Anatomical Diagnosis.*—Verrucous endocarditis of mitral and aortic valves; aortic stenosis and insufficiency; cardiac hypertrophy and dilatation; old and recent fibrous pericarditis; fibrous pleurisy (left). Heart weighed 810 gm. The tricuspid orifice is slightly thickened and along its line of closure there are scattered numerous small verrucae varying from 1 to 3 mm. in size. The mitral valve is dilated and presents numerous verrucae; mitral ring calcified; the cusps of the aortic valve are markedly thickened and along the free edge of the cusps are numerous verrucous projections.

*Microscopical Examination. Heart.*—Section stained with methyl-green pyronin. Great numbers of Aschoff bodies and diffuse fibrosis of the myocardium. The bodies are found in the wall of the left ventricle, left papillary muscles, interventricular septum, about the calcified mitral ring, beneath the endocardium, and in the mitral valve, but not in the auricles.

Three main types of this lesion occur: (1) Collections of a few typical cells not more than eight to ten in number, closely packed against one another, immediately around small blood vessels. (2)

<sup>3</sup> Case 1 was studied through the kindness of Dr. Alfred Meyer, of Mount Sinai Hospital.

Large collections of cells, numbering thirty or forty, arranged in a loose network of fibroblasts and young connective tissue cells. These occur mainly about the arteries, usually those of large or medium size, and beneath the endocardium. Some of the collections resemble a rosette in form, but most of them are fusiform accumulations in the adventitia of the blood vessels, and many may be found on both sides of the blood vessel as though completely encircling it. (3) A small number of perivascular foci made up of dense fibrous tissue with a few large typical giant cells at their center.

The greatest number of the bodies are found about the mitral ring, where in the midst of a dense fibrous tissue, which is infiltrated with calcium, are found typical discrete collections and large masses of scattered cells. Many plasma cells and large mononuclear leucocytes and a few polymorphonuclear leucocytes are found here and elsewhere about the bodies, together with numerous fibroblasts which take a bright red stain somewhat different from that of the giant cells, some of which are drawn out, flattened, or fusiform in shape. The mitral valve itself shows a number of the bodies mainly of the second type described, and in addition a dense fibrosis, fibroblasts, and mononuclear and plasma cells. The blood vessels everywhere, especially the small and medium sized ones, show a moderate intimal thickening while in some places there seems to be a narrowing of the lumen opposite the Aschoff bodies.

*Case 2.*<sup>4</sup>—R. H., age 17 years; negro, male. Admitted to the City Hospital, February 17, 1913; died, February 23, 1913. Patient complained of pain and swelling of ankles and knees. *Past history.*—Rheumatism five years ago with swelling of joints. *Present illness.*—Began about a week before admission with pain and swelling of ankles, later of both knees.

*Autopsy.*—Fibrous hypoplastic aorta; lymphatic hyperplasia of spleen and mesenteric lymph nodes; persistent thymus; fibrous pericarditis. *Heart.*—Weight 300 gm. The whole heart is encased in a dry, fibrinous exudate; valves normal. Left ventricle measures 9 mm. in thickness. On section the myocardium is normal in color, and shows no areas of sclerosis. Coronary arteries normal; aorta narrow, and free from arteriosclerosis.

*Microscopical Examination. Heart.*—Section stained with methyl-green pyronin. The heart contains a great number of Aschoff

<sup>4</sup> Case 2 was studied through the kindness of Dr. John Larkin, of the City Hospital, New York.

bodies, there being several in almost every microscopic field. Practically all the bodies are large, containing twenty to forty cells; they are arranged beneath the endocardium, in the adventitia of the blood vessels, and between the muscle fibers apparently independently of blood vessels. About half the cells are of the large multinuclear type, of which some contain five or six nuclei. The arrangement is loose, not densely packed together as in case 1. Between some of the cells is a pink staining matrix which contains many fine fibrils (fibrin?). In other bodies the cells are separated by a fibroblastic kind of tissue.

*Case 3.*<sup>5</sup>—J. S., age 23 years; white, female; married. Admitted to the City Hospital, January 23, 1913; died, March 5, 1913. Patient complained of headache, pain in joints and chest. *Past history.*—Scarlet fever when a child; typhoid one year ago. *Present illness.*—Began about three months before admission. The patient died forty-one days after admission to the hospital. Cardiac murmur remained present and unchanged with the exception of pericardial friction rub which appeared for a few days and then disappeared. Patient developed dullness on both sides of the chest posteriorly about two weeks before death. The chest was tapped and large quantities of clear fluid were removed on two occasions. This fluid was cultured twice, and cultures and spreads were negative. Three blood cultures were taken and all were negative. Wassermann reaction was negative.

*Autopsy.*—Only a partial autopsy was obtained. *Heart.*—Slightly hypertrophied and showed a slight chronic thickening of all its valves, and a few typical small pin-head verrucæ along their edges.

*Microscopical Examination.*—Section stained with methyl-green pyronin. The heart contains only a few typical Aschoff bodies in the adventitia about several medium sized or larger arteries. The bodies contain from six to ten typical cells, some of which are large and multinuclear; most of them are slightly larger than large mononuclear leucocytes and contain a single nucleus. The cells which are chiefly elongated have no definite arrangement and are separated from one another by the connective tissue of the adventitia; they lie with their long axis parallel to the direction of the blood vessel.

*Case 4.*<sup>6</sup>—M. W., age 17 years. Admitted to Mount Sinai Hospital, June 16, 1913; died, June 23, 1913. Onset of illness six weeks before admission, with headache, epistaxis, vomiting, generalized pain. Satisfactory history unobtainable. *Streptococcus mitis (viridans)* was recovered by blood culture.

<sup>5</sup> Case 3 was studied through the kindness of Dr. John Larkin, of the City Hospital, New York.

<sup>6</sup> Case 4 was studied through the kindness of Dr. J. Rudisch, of Mount Sinai Hospital.

*Autopsy. Anatomical Diagnosis.*—Subacute aortic and mitral bacterial endocarditis; verrucous mitral endocarditis and mitral stenosis; infarcts in the spleen and kidney; embolic glomerular lesions. *Heart.*—Normal in size; wall of left auricle increased in thickness; mitral valve is narrowed; the flaps are thickened and fibrous and continue downward onto the chordæ tendinæ for a slight distance, forming a funnel-shaped orifice, the free edge ending in a slight projecting shelf beyond the chordæ. Along the free edge of this shelf-like projection, very close to one another, are many smooth, pin-head verrucæ which are translucent in appearance. They are firm and crush with difficulty. On the ventricular surface of the aortic flap of the mitral valve are two large pedunculated cauliflower-like vegetations about half a centimeter in width and projecting for a distance of one centimeter. They are mixed red and gray in color and are friable. The cusps of the aortic valve show a slight irregular fibrous thickening. The ventricular surface of the cusps contains numerous small warty vegetations similar to those around the base of the large mitral vegetation. One of the cusps of the aortic valve, which is covered by vegetations, comes in contact with the large mitral vegetation when in the closed position.

Smears from the large mitral vegetation and those on the aortic valve show a moderate number of minute gram-positive cocci, which in culture proved to be *Streptococcus mitis (viridans)*. Smears and cultures, from crushed verrucæ on the mitral valve, were negative.

*Microscopical Examination.*—Sections stained with hematoxylin and eosin. Section of auricular-ventricular junction, mitral valve, left ventricle, and papillary muscle. The section shows diffuse round cell infiltration, slight edema, and patchy increase in interstitial tissue. Within the fibrous patches and between the muscle fibers, focalized collections of round cells giving the same picture as the lesions produced experimentally by Bracht and Wächter occur, including atrophy and degeneration of muscle fibers. In the adventitia of a number of the large and medium sized vessels and also in the myocardium between the muscle fibers are seen a moderate number of irregular nodular collections of cells, three to four times the size of large mononuclears, of which some are elongated and others multinuclear. The nuclei are vesicular, the protoplasm is finely granular, while between some a material which is either coagulated serum or fibrin occurs. The mitral valve shows fibrosis, slight round cell infiltration and small, irregular projections corresponding to the verrucæ, which are composed of more recent fibrous tissue. The blood vessels are thickened and show round cell infiltration. }

In sections stained with methyl-green pyronin the nodular collections of cells just described take a brilliant dark red stain and are

seen to be typical Aschoff bodies. Others occur beneath the endocardium, and a few in the papillary muscle. The verrucæ at the edge of the mitral valve are made up of young connective tissue network. Throughout these small rounded projections are seen brilliant, dark red staining cells, the protoplasm of which is either homogeneous or finely granular. The cells are either fusiform, and three to four times the size of large mononuclear cells, or present long, interlacing, streamer-like processes. The nuclei are large and poor in chromatin. The first cells are suggestive of the cells of the Aschoff bodies, the latter having the appearance of fibroblasts.

*Case 5.<sup>7</sup>*—F. B., age 10 years. History of chorea at the age of 7; three attacks before the present admission; child died with symptoms of meningitis; clear cerebrospinal fluid.

*Autopsy.*—The pericardium is densely adherent to the sternum and when opened a large quantity of seropurulent fluid escaped. Parietal and visceral layers are rough, granular, and congested. *Heart.*—Both ventricles are hypertrophied; near the free margins of the tricuspid, mitral, and aortic valves are numerous small vegetations.

*Microscopical Examination.*—Section stained with methyl-green pyronin. The heart contains a few atypical Aschoff bodies. Many of the medium sized arteries show irregularly distributed thickening of the intima. In these arteries, diffusely scattered through the intima and extending into the adventitia are found cells resembling young fibroblasts and proliferating endothelial cells; but in the adventitia many of the cells have the typical appearance of Aschoff cells of which a number contain two or three nuclei.

*Case 6.<sup>8</sup>*—Female, age 12 years. Two weeks before the present illness the patient had tonsillitis and later complained of pain in arms and legs. One week later symptoms of chorea developed; movements became extremely violent and child became delirious, passing into coma and dying forty-eight hours later. Examination showed a few fibrous nodules along the tendons of the palms of the hands.

*Autopsy. Anatomical Diagnosis.*—Acute vegetative mitral endocarditis; bronchopneumonia of right lung; congestion of the pia mater. *Heart.*—Weight

<sup>7</sup> Case 5 was studied through the kindness of Prof. E. L. Opie, of George Washington University, and Prof. John Howland, of Johns Hopkins Medical School.

<sup>8</sup> Case 6 was studied through the kindness of Prof. E. L. Opie, of George Washington University, and Prof. John Howland, of Johns Hopkins Medical School.

172 gm.; endocardium normal, except at the line of closure of the mitral cusps on which is a continuous line of firmly attached vegetations.

*Microscopical Examination.*—Section stained with methyl-green pyronin. The heart contains a moderate number of typical Aschoff bodies present only in the adventitia of the arteries. They are made up of collections of six to forty cells which in a few instances are densely packed. In most cases the cells occur in the midst of the connective tissue, and are slightly separated from one another. The cells, of which one third are mononuclear, appear to be young, and many of them resemble fibroblasts.

*Case 7.<sup>9</sup>*—E. S., age 16 years. Admitted to Mount Sinai Hospital, Sept. 28, 1907; died, Oct. 27, 1907. Diagnosis, acute endocarditis. Gave a previous history of measles; chorea four years ago; no articular symptoms or sore throat; no history of rheumatism. For two months before admission had dyspnea and palpitation on exertion; cough, worse at night; headache.

*Autopsy. Heart.*—Enlarged; small areas of thickening of pericardium; walls of ventricles and auricles markedly hypertrophied. All cavities considerably dilated. Hypertrophy of papillary muscles of left ventricle. Mitral valve admits two fingers. All flaps markedly thickened. Free edges of flaps show irregular small verrucous vegetations. On the wall of the left ventricle, about two centimeters below the aortic orifice, there is a line of minute vegetations. All cusps are markedly thickened and retracted; about the free edge there are verrucous vegetations, some of which are five millimeters in diameter. The tricuspid valve admits two fingers; many vegetations occur along the thickened free edges of the cusps. The heart shows myocarditis, particularly in the left ventricle.

*Microscopical Examination.*—Section stained with methyl-green pyronin. The heart shows throughout numerous Aschoff bodies, most of which are large and contain as many as a hundred cells, and large, irregular areas of fibrosis in which a moderate diffuse round cell infiltration and a few polymorphonuclear leucocytes exist. The Aschoff bodies occur almost entirely in the adventitia of the medium sized and small arteries and in the midst of the areas of fibrosis. The bodies are loose collections of typical cells largely separated from one another by connective tissue fibers and containing in the center between the cells pink staining granular material. Some of the cells contain six nuclei.

<sup>9</sup> Case 7 was studied through the kindness of Dr. J. Rudisch, of Mount Sinai Hospital.

## CASES IN WHICH ASCHOFF BODIES WERE NOT PRESENT.

We have examined the hearts of fourteen cases of subacute bacterial endocarditis,<sup>10</sup> of which disease descriptions have been given by Osler, Schottmüller, Horder, Libman, and others. The specimens were obtained from cases which Dr. Libman has reported. Blood cultures during life were positive in all, the invading organism being *Streptococcus mitis*. Ten of the cases gave no history of rheumatism. In none of these could Aschoff bodies be demonstrated. In only one of the four cases in which a history of rheumatism existed did we find any evidence of Aschoff cells. These cells characteristic of the bodies were few and occurred in the fibrous scars about the small vessels. This patient had suffered from an attack of acute articular rheumatism eleven years and again one year before death. Two other cases had "rheumatism" five and seven years, respectively, before death. The fourth patient had "rheumatism" with valvular disease fourteen years before admission. At the time of admission he had joint pains and was evidently suffering from a superadded bacterial infection involving his previously damaged valves. It is difficult to distinguish the arthritic manifestations in bacterial endocarditis from those of acute rheumatic fever. We have also examined the hearts of five cases of subacute bacterial endocarditis in the bacteria-free stage, as first described by Dr. Libman. Only one gave a history of "rheumatism," the attack antedating death by five years. None showed Aschoff bodies. In all these hearts lesions occurred similar to those produced experimentally by Bracht and Wächter with *Streptococcus mitis*. We have also examined with negative results the hearts of five patients that had had chronic valvular disease without rheumatism. In five cases of acute streptococcus endocarditis, one case of suppurative pericarditis accompanied by pneumococcus endocarditis, two cases of gonococcus endocarditis, and three cases of staphylococcus endocarditis, we failed to find the presence of Aschoff bodies in the heart.

## CONCLUSIONS.

1. In rheumatic myocarditis, foci, termed submiliary nodules of Aschoff, are present which are characteristic of the rheumatic infection.

<sup>10</sup> A large series of cases is being studied and will be reported later.

2. They are most frequently found in the walls of the left ventricle, the auricles usually escaping.

3. The nodules were found in three cases of chorea without joint manifestations, proving the close relation of this condition to rheumatism.

4. They were absent in fourteen cases of subacute bacterial endocarditis due to *Streptococcus mitis*.

5. They were not found in infections of the endocardium with the gonococcus, staphylococcus, streptococcus, or pneumococcus.

6. Even in the absence of a rheumatic history we believe, in accordance with Fraenkel, that the presence of Aschoff bodies signifies a previous rheumatic infection.

7. Aschoff bodies are not always found in rheumatic carditis, where the infection antedates death by a long period, but the healed remains, represented by sclerotic patches ("Schwielen"), are present.

8. We suggest that the cases of arthritis characterized by the presence of the submiliary nodules of Aschoff in the myocardium be placed in one group and called for the time being "rheumatism"; and the cases with articular manifestations, yielding positive bacteriological findings and no Aschoff bodies, should be classified according to the infecting microorganisms concerned, and not as rheumatism.

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#### EXPLANATION OF PLATES.

##### PLATE 46.

FIG. 1. Aschoff bodies, situated in the adventitia of a medium sized artery, showing typical morphology. Stained with hematoxylin and eosin.  $\times 80$ .

FIG. 2. Aschoff body, situated in the adventitia of a small artery. Stained with methyl-green pyronin.  $\times 160$ .

##### PLATE 47.

FIG. 3. Left auricle with subendocardial Aschoff bodies and thickened endocardium. Stained with methyl-green pyronin.  $\times 40$ .

FIG. 4. Aschoff bodies in interstitium of left ventricle. Stained with methyl-green pyronin.  $\times 72$ .

FIG. 5. Aschoff body, showing multinuclear cells. Stained with methyl-green pyronin.  $\times 224$ .

##### PLATE 48.

FIG. 6. Aschoff body in interstitium of left ventricle, from case of chorea. Stained with methyl-green pyronin.  $\times 104$ .

FIG. 7. Interstitial lesion of fibrous type (Bracht and Wächter) in heart showing both subacute bacterial endocarditis and verrucous endocarditis. Stained with methyl-green pyronin.  $\times 16$ .



FIG. 1.

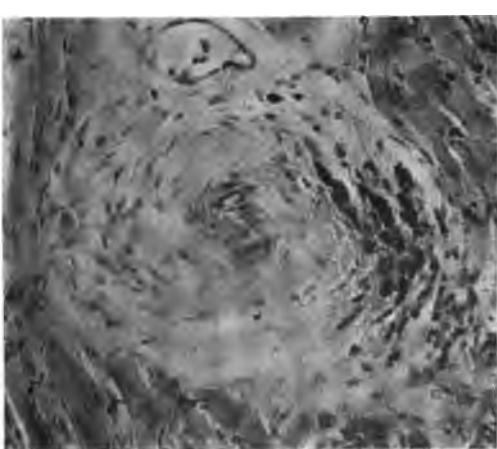


FIG. 2.

(Thalhimer and Rothschild: Nodules of Aschoff.)



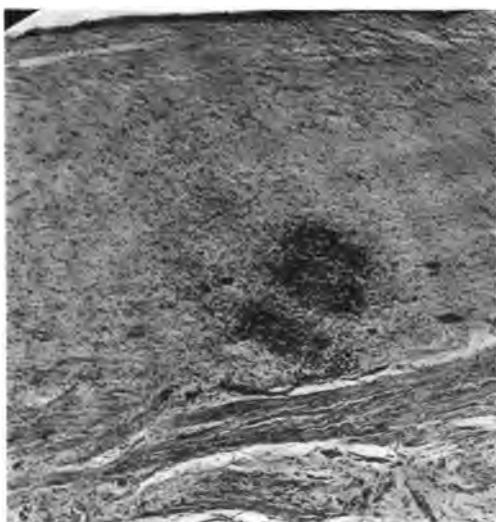


FIG. 3.

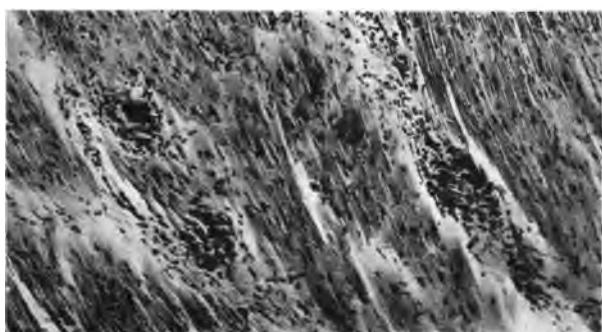


FIG. 4.

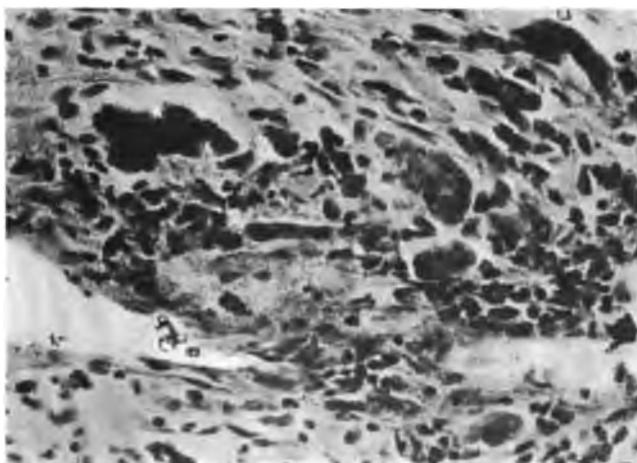


FIG. 5.

(Thalhimer and Rothschild: Nodules of Aschoff.)



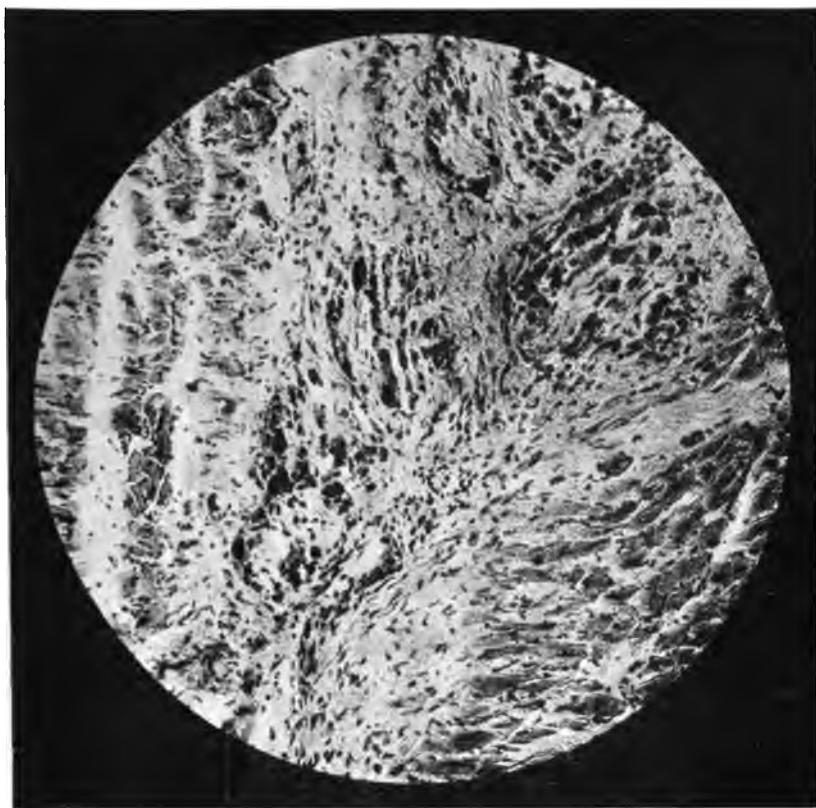


FIG. 6.

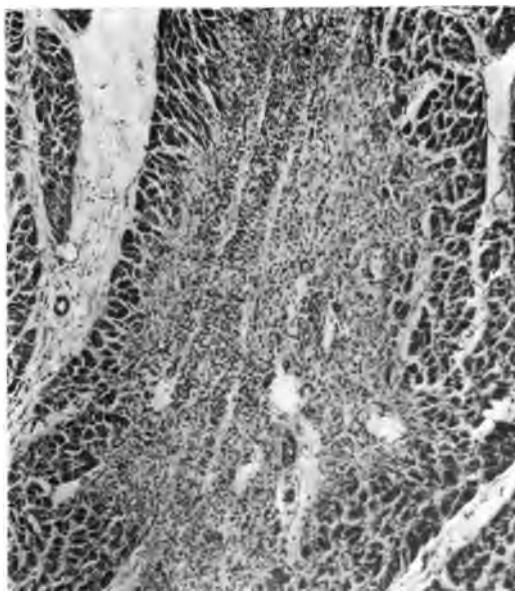


FIG. 7.

(Thalhimer and Rothschild: Nodules of Aschoff.)



MOUNT SINAI HOSPITAL

## EXPERIMENTAL FOCALIZED MYOCARDIAL LESIONS PRODUCED WITH STREPTOCOCCUS MITIS.\*

By WILLIAM THALHIMER, M.D., AND M. A. ROTHSCHILD, M.D.  
(From the Pathological Laboratory of the Mount Sinai Hospital, New York.)

PLATES 49 AND 50.

The involvement of the heart in the pathological processes of acute rheumatic fever has long been known. It takes the form of endocarditis, pericarditis, and myocarditis, either alone or in combination. Of these the submiliary myocardial nodules of Aschoff alone seem to be specific. Because of the resemblance of acute rheumatism to diseases of an infectious nature the search for the etiological agent of rheumatism and its associated conditions has centered about a bacterial cause.

The literature on this phase of the subject has been reviewed by Poynton and Paine, Pribram, Sanderson, Menzer, de Vecchi, and others. The different observers may be classified into two groups according to the scheme of de Vecchi: first, those who using a perfect technique failed to cultivate bacteria from the blood or joints of these cases and who do not believe in a bacteriological etiology; and second, those who have succeeded in recovering bacteria. Different bacteria have been found by the observers belonging to the second group. Some, mainly the older investigators, believe that rheumatism is caused by common, pyogenic bacteria. Leyden, Poynton and Paine, Rosenow, and others have recovered a diplostreptococcus from cases which they have classified as rheumatism. Still another group, mostly of the French school, have cultivated a Gram-negative, anaerobic bacillus. Finally, other authors have considered this disease a mixed infection of cocci and bacilli, perhaps not even specific.

Our interest in this subject was aroused by the statements of Meyer, Shaw, and Major concerning the identity of the cultural characteristics of *Streptococcus rheumaticus* (of Leyden, Triboulet, Meyer, Wassermann, Poynton and Paine, and others) and *Streptococcus viridans* (Schottmüller), better named, in our opinion, *Streptococcus mitis*. The last named organism has been isolated in this laboratory from the blood of cases of subacute bacterial endocarditis which have been studied extensively by Dr. Libman.<sup>1</sup>

\* This work was done under the tenure of a George Blumenthal, Jr., Fellowship. Received for publication, February 3, 1914.

<sup>1</sup> The cultural characteristics of these organisms will not be discussed here, since studies of this entire group of streptococci will soon be published by Drs. Libman and Aschner.

## CHAPTER VI

### EXPERIMENTAL FOCALIZED MYOCARDIAL LESIONS.

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Poynton and Paine and Rosenow are the leading advocates of the theory that *Streptococcus rheumaticus* is the logical cause of rheumatism. The former base their arguments on two main facts: first, the recovery of this variety of diplostreptococcus from the joints, urine, tonsils, subcutaneous nodules, etc., from the cases classified by them as rheumatism; and second, the production in rabbits of endocarditis, pericarditis, myocarditis, arthritis, chorea (?), etc., which they consider the counterpart of the manifestations of rheumatism. Numerous workers have shown, however, that other types of bacteria can produce carditis. The above named authors have not definitely shown the identity of the experimental and the human lesions. Horder studied a comparatively large series of cases of acute rheumatic fever and malignant endocarditis, and whereas he was able to recover by blood culture a diplostreptococcus, which appeared to him to be identical with *Streptococcus rheumaticus*, from 90 per cent. of the cases of malignant endocarditis (*Bacillus influenzae*, etc., from the remainder), he was unable to recover any bacteria from the blood or joint exudate of cases of acute articular rheumatism. He came to the conclusion that the contradictory results of the various observers were not due to insufficient bacteriological technique, but rather to a discrepancy in classification, the cases from which the *Streptococcus rheumaticus* was isolated being instances of malignant endocarditis and not rheumatism, or, as they are called by Poynton and Paine, "malignant rheumatism."

Rosenow has produced similar lesions in rabbits injected intravenously with large doses of an organism resembling *Streptococcus viridans* (Schottmüller), isolated by blood culture from cases of chronic infective endocarditis, and by similar organisms recovered from the tonsils. Rosenow and Coombs have described myocardial lesions in these rabbits which they consider similar to Aschoff bodies, but which in our opinion do not resemble these bodies. Rosenow has also recovered three types of a diplostreptococcus from the blood and joint exudates of a series of cases of rheumatism. These organisms, he claims, when recently isolated have on intravenous injection in rabbits an affinity for the joints though they also cause cardiac lesions. Rosenow considers this work a confirmation of that of Poynton and Paine, although in his cultures the streptococci grew in the original culture only in small numbers and under partial anaerobic conditions, whereas the latter workers recovered their organisms in large numbers and under ordinary aerobic conditions.

Bracht and Wächter, working with a similar type of *Streptococcus mitis*, caused by intravenous injection in rabbits definite myocardial lesions identical with those later described in this paper. They considered these lesions entirely different from Aschoff bodies. De Vecchi, who looks with scepticism on the importance of all bacterial findings in their primary relation to rheumatism, made a number of experiments by injecting blood from rheumatic cases into several species of animals. He described peculiar perivascular myocardial lesions containing a peculiar type of large mononuclear leucocyte, but having no particular resemblance to Aschoff bodies. His work, however, is not convincing.

Coombs, experimenting with *Streptococcus rheumaticus*, claims to have produced Aschoff bodies in rabbits, but the lesions he describes resemble more those described by Bracht and Wächter than they do those of Aschoff. Jackson produced focalized myocardial lesions in rabbits by intravenous injection of strepto-

cocci isolated from the sore throat epidemic in Chicago. These animals developed arthritis and some of them endocarditis. The myocardial lesions are those of a combined exudative and productive inflammation and have some points of resemblance to those produced by Bracht and Wächter. They do not conform to Aschoff bodies.

#### EXPERIMENTAL.

The present work was undertaken to ascertain the cardiac lesions produced by intravenous injections of *Streptococcus mitis*, and not to investigate this organism from the point of view of the etiological agent of rheumatism. For comparison inoculations were made with *Streptococcus rheumaticus*, *Streptococcus hemolyticus*, and a streptococcus isolated from cases of epidemic sore throat. Since Poynton and Paine, Rosenow, and others recovered a diplostreptococcus from cases of acute rheumatic fever, and Jackson and Coombs claim to have produced lesions similar to the submiliary myocardial nodules of Aschoff by inoculating streptococci, attention has been directed to the relation, if any, between the myocardial lesions arising in our experiments and the Aschoff bodies.

*Organisms Used.*—Nine different strains of *Streptococcus mitis* were used (Nos. 4,110, 4,147, 4,170, 4,206, 4,275, 4,342, 4,357, 2,524, and organism C). Organism C was isolated from the tonsils of a case of phlegmon of the neck; the other organisms were obtained by blood culture from cases of subacute bacterial endocarditis studied by Dr. Libman. All except No. 2,524 were recently isolated strains, often only one or two cultures removed from the original blood cultures, and resembled the *Streptococcus viridans* of Schottmüller. The growth on agar adhered to the surface of the medium, while in bouillon the organisms grew in clumps and adhered to the sides of the tube. Organism 2,524 was an old strain and had been in the laboratory for three years, being transplanted on glucose serum agar every four days.

The miscellaneous cultures consisted of five strains of *Streptococcus rheumaticus*, and three strains of epidemic streptococci.<sup>2</sup>

<sup>2</sup> We are indebted to Dr. Winslow, of the American Museum of Natural History, New York, for No. 34 (Poynton and Paine), No. 259 (Beattie), and No. 399 (Linz), and to Dr. Rosenow, of the Memorial Institute, Chicago, for Nos. 735 and 736. Dr. Rosenow kindly furnished us with three strains of streptococci isolated from the Chicago sore throat epidemic, Nos. 211, 214, and 233, which had been studied by Jackson.

The organisms were grown on serum agar, serum glucose agar, human blood agar, and plain bouillon. Thick saline suspensions were made of the twenty-four hour agar cultures and the growth in bouillon was concentrated by centrifugalization. In some instances rabbits were inoculated with blood containing innumerable bacteria taken from another animal with vegetative endocarditis.

The dose varied from the twenty-four hour growth on one slant agar tube to that on two Blake bottles, or the centrifugalized sediment of a twenty-four hour growth in from 10 to 100 cubic centimeters of bouillon. Rabbits received from one to sixteen injections at intervals of from three days to several weeks.

The rabbits were of different ages, young, half grown, or old, and came from several stocks, in the attempt to rule out any special susceptibility or resistance in any one strain. One monkey was also employed.

#### STREPTOCOCCUS MITIS.

Forty-two rabbits were inoculated with *Streptococcus mitis*. All showed the microscopic lesions described below. Four were killed 1, 3, 4, and 7 days after injection, and a few died within the first week, but most lived for a long period, and one for as long as six months. Many of the animals developed arthritis and became emaciated, and those that lived longest developed a scaly condition of the skin of the face and paws. Only three rabbits (7 per cent.) developed vegetative endocarditis, a much smaller proportion than that reported by Rosenow. Different media, and in some instances organisms only two generations removed from the original blood culture, were used. Rabbit blood agar was not used;<sup>3</sup> and whether this difference is responsible for the smaller percentage of endocardial localizations can only be conjectured.<sup>4</sup> Nine rabbits (21 per cent.) developed hemorrhages in the valves.<sup>5</sup> The only rabbits that showed a bacteremia were those with vegetative endocarditis.

<sup>3</sup> At present this work is being repeated by one of us (Thalhimer) in conjunction with Celler, using organisms grown on rabbit blood agar.

<sup>4</sup> The same result was observed by Drs. Libman and Celler, in 1903 (unpublished), who inoculated a large number of rabbits, of which a small percentage developed endocarditis.

<sup>5</sup> Hemorrhages in the cardiac valves are considered by Rosenow to be the earliest stage of an endocarditis caused by *Streptococcus mitis*.

Even those dying a few days after injection showed sterile blood at autopsy. In a few animals blood cultures were taken from the ear vein of the opposite ear to that injected at intervals of 1, 5, 10, 30, and 60 minutes after injection. The number of colonies decreased rapidly with the lengthening of the interval and none were found after ten minutes had elapsed.

The urine of a considerable number of the rabbits was examined. In none did indications of spontaneous albuminuria occur during the period of observation before injection.

Many of the animals developed after injections a transitory albuminuria with casts, and several showed this condition constantly after many injections. Two animals became anuric for forty-eight hours, one just before death. During the anuria the animals were drowsy, but when a sudden noise was made, they would suddenly jump in a spasmodic fashion as though slightly uremic.<sup>6</sup> Renal lesions, which are being investigated further, were found in this series of animals.

A monkey received seven injections of the contents of a twenty-four-hour growth on four Blake bottles of serum glucose agar; it showed myocardial lesions identical with those in the rabbit.

*Myocardial Lesions.*—In most cases the hearts showed no macroscopical lesions, except those mentioned; but in a few instances grayish streaks existed in the myocardium. Viewed microscopically a progressive type of lesion was present, depending on the number of injections and the length of time the animal lived.

The earlier stage of the lesion is represented by small areas including only a few muscle fibers showing slight degenerative changes, and between and about them a few infiltrating lymphocytes, large mononuclear leucocytes, occasional plasma cells, and rarely a few polymorphonuclear leucocytes. In animals receiving many injections the areas are larger, and in them the degenerating fibers show at times marked fragmentation, while the surrounding fibers are normal. The degenerating muscle fibers take a slightly more intense eosin stain than normally and the fibrils disappear early, leaving a homogeneous, finely granular protoplasm with small

<sup>6</sup> This condition has been reported by several observers, and may have been interpreted as chorea in rabbits.

and large vacuoles. Some of the fibers are swollen, but most are atrophic. The nuclei are irregular and either pale and vesicular, or small and pyknotic, and are not proliferating.

In addition to these more common lesions some of the hearts which show grayish myocardial streaks show microscopically areas of complete necrosis of the muscle fibers with calcareous infiltration and a slight round cell inflammatory reaction about them. With the atrophy of the muscle fibers, the interstitial tissue stands out more prominently and very early begins to proliferate, fibroblasts appearing. These areas are irregular in size and shape and are usually elongated, with the long diameter parallel to the muscle fibers. From this stage on the lesions are proliferative rather than infiltrative leucocytic or degenerative. In none of the areas are many degenerating muscle fibers seen, but fibroblasts soon become prominent and develop rapidly to a fibrous stage. Coincidently the round cell infiltration decreases until the result is an area of fibrous tissue surrounded by healthy muscle fibers and containing a few leucocytes.

Not all the fibrous areas are circumscribed; many are diffuse, their fibers running parallel to those of the myocardium, apparently having replaced the latter. Some of the focalized lesions have a close relation to the small and medium sized blood vessels, but many lie scattered through the myocardium, the greatest numbers occurring near the base of the ventricles and in the papillary muscles. The small blood vessels in some cases show proliferation of the intima or the connective tissue immediately about them. Occasionally hyaline thrombi are found, but they do not occur regularly and appear to have no relation to the lesions. The lesions themselves show no predilection for a subendocardial situation nor for the bases of the cardiac valves; no proliferation of the endothelium of the endocardium or valves occurs. No multinuclear cells such as are described by Jackson, or peculiar cells with large vesicular nuclei and scanty protoplasm as described by de Vecchi, are present. With the methyl-green pyronin stain, on alcohol-fixed material, the fibroblasts, and in some hearts the endothelium of the blood vessels, stained a bright red, as do some of the degenerating muscle fibers. No structures at all resembling the submiliary myocardial bodies of

Aschoff are found, and the red staining of the fibroblasts, etc., with methyl-green pyronin has an entirely different tint from that taken by the cells of Aschoff bodies. The latter stain a deep, rich red and the fibroblasts a bright red, not so deep and with a highly refractile quality, giving them an appearance which for lack of a better term we call translucency. In none of the hearts, although carefully searched for, were bacteria found either in association with the lesions or elsewhere, except in those hearts where vegetative endocarditis was present. In them streptococci were found diffusely scattered in small clumps in the capillaries between normal muscle fibers and also in some of the lesions, but not in all the lesions in these three hearts.

#### STREPTOCOCCUS RHEUMATICUS AND EPIDEMIC STREPTOCOCCI.

The hearts of the eleven rabbits injected with *Streptococcus rheumaticus* showed changes identical with those described above. One rabbit (No. 13) developed a transient bacteremia, and at autopsy the only gross lesions in the heart were a few whitish streaks over the endocardium of the left ventricle. This was the only animal injected with an anhemolytic type of streptococcus which developed a bacteremia in the absence of vegetative endocarditis. Seven rabbits were injected with streptococci from epidemic sore throat and developed the usual pyogenic type of lesions caused by *Streptococcus pyogenes*; they died in the course of a few days. The lesions consisted of extensive degenerative and destructive processes associated with leucocytic infiltration, mainly polymorphonuclear, and with the presence of great numbers of cocci. A few fusiform areas resembling somewhat those described by Jackson were present, but were devoid of giant cells and in no way similar to Aschoff bodies. One rabbit that had been injected with six small non-lethal doses of this streptococcus and lived for ninety-eight days developed multiple arthritis and showed microscopically in the myocardium focalized lesions which occupied a position between the type of lesions produced by *Streptococcus mitis* and that caused by *Streptococcus pyogenes*, but resembling the former more closely.

## ILLUSTRATIVE PROTOCOLS.

*Rabbit 18.*—White, full grown. Dec. 22, 1912. Intravenous injection of one serum glucose agar slant of organism 4,110. Dec. 30. Two slants injected Jan. 13, 1913. Two slants injected. Jan. 18. Swelling of left fore leg. Feb. 19. Two slants injected. Feb. 20. Dead.

*Autopsy.*—Heart normal. Diffuse pneumonia of both lungs. Spleen three times normal size; brown in color. Kidneys enlarged. In the right kidney there is a large subcortical hemorrhage. Surface roughened; many depressions surrounded by hemorrhagic zones. One large recent infarct extending from cortex to medulla. Joints normal.

*Microscopical Examination. Heart.*—Stained with hematoxylin and eosin. The wall of the left ventricle shows a slight grade of diffuse increase of interstitial tissue which in places forms small collections mostly about blood vessels. Near these accumulations of interstitial tissue the muscle fibers surrounded by this tissue stain palely and taper out and are lost in the midst of the connective tissue. A few round cells are present. Other parts show small focalized collections of fibrous tissue, but no diffuse increase in interstitial tissue. Methyl-green pyronin stain gives the same result as above.

*Rabbit 36.*—White; weight 2,020 gm. Apr. 9, 1913. Intravenous injection of organism 4,110. Apr. 10. Urine normal. Injected one and a half serum glucose agar slants. Apr. 11. Albumin present in urine; few hyaline casts. Apr. 12. Albumin but no casts. Apr. 13. Urine normal. Apr. 16. Trace of albumin, many pus, few red cells. Apr. 18. Injected twenty-four hour tube of blood bouillon culture. Apr. 19. Urine: many pus, and few red cells, no casts. Apr. 27. Three cultures injected. Apr. 29. Urine: albumin, few pus and red cells. Apr. 30. Urine: trace of albumin, few granular casts. Culture: ten drops of blood gave several colonies. May 1. Albumin, few granular and hyaline casts. Culture: 2,000 colonies. May 2. Culture: ten drops of blood gave 4,000 colonies; typical *Streptococcus mitis*. Trace of albumin, moderate number of granular casts, few red cells; clump of pus cells. May 7. Albumin and casts. Culture: six drops of blood gave 5,000 colonies. May 8. No urine in last twenty-four hours. Not eating. Spontaneous twitching and shaking movements. May 9. Poor condition. 11 A. M., 4 c.c. of smoky urine; 12.30 P. M., died. Urine obtained post mortem showed much albumin; large numbers of epithelial casts, and few hyaline and granular casts. Culture gave *B. coli*. Blood culture: 1 c.c. gave innumerable colonies. 5 c.c. of pleural fluid gave 100 colonies; pericardial fluid gave few colonies. Knee joint: 30 colonies from one loop of fluid. Microscopical examination of fluid showed a moderate number of polynuclear cells and a few degenerated forms of streptococci. Endocardiac vegetations and pure cultures of streptococcus.

*Autopsy.*—Pericardium contains about 1 c.c. of clear serous fluid. Heart dilated; moderate number of minute petechial hemorrhages over the epicardium, along the blood vessels. Tricuspid valve contains diffuse red hemorrhages in

septal leaflet beneath which is a slight projection of the interventricular septum pushing the valve before it. Left auricle: projecting from the mitral ring and practically occluding it are grayish, granular, cauliflower-like vegetations, springing from the entire auricular surface of the mitral flaps to within a short distance of the insertion, but not upon the auricular wall. The vegetations consist of three or four masses, 6 to 7 mm. in diameter, cauliflower-like in appearance, with a dry, finely granular surface. They arise from the auricular surfaces of the flaps; the narrowing of the mitral orifice is caused by the vegetations and not by constriction of the ring. The vegetations pass to the chordæ of the anterior flap as far as the papillary muscle, but none of the chordæ are eroded across. Several minute vegetations affect the anterior papillary muscle near the insertion of the chordæ. The ventricular surface of the anterior flap of the mitral valve is opaque and wrinkled. Left ventricle: the septum just below the aortic valve contains transparent pin-head vegetations about 1 cm. in width. The aortic valve is normal. Chest: each pleural cavity contains 8 to 10 c.c. of serosanguinous fluid. The lungs did not collapse when the chest was opened. On the surface are many bright red areas from 1 to 4 mm. in width beneath which are areas of red hepatization. Peritoneal cavity: slight excess of fluid; parietal and visceral peritoneum show minute petechial hemorrhages. The liver is normal in size and the lobules stand out distinctly, the centers being translucent and the peripheries opaque and fatty. Spleen: the spleen was greatly enlarged, almost black in color, and on the convex surface is a linear scar-like depression, about 1 cm. in length and 2 mm. in depth. The kidneys are normal except for a few subcapsular petechial hemorrhages. The bladder was empty. The large joints contain a small amount of sticky, gelatinous, opalescent material, and few petechial hemorrhages occur in the synovial membranes. The brain shows numerous petechial pial hemorrhages over the convexity of the brain.

*Microscopical Examination.*—Stained with hematoxylin and eosin. The heart shows slight diffuse leucocytic infiltration as well as large numbers of lesions similar to those described by Bracht and Wächter. The lesions exhibit fragmented nuclei, while the muscle fibers in and about them show an extreme degree of degeneration and fragmentation which are absent from the muscle fibers elsewhere. Some of the lesions are older than others and in them fibroblastic replacement of the muscle fibers has occurred. The methyl-green pyronin stain brings out in addition a few plasma cells. The Gram-Weigert stain discloses streptococci in short chains and small groups in the capillaries between the muscle fibers without reaction about them and not associated with the focal lesions.

*Rabbit 13.*—White, full grown. Injected with organism 34, *Streptococcus rheumaticus* (Poynton and Paine). Dec. 12, 1912. Received one tube of serum glucose agar culture. Dec. 20. The same. Dec. 26. About twenty-five colonies in ten drops of blood. Dec. 30. Received two tubes of the culture. Jan. 13, 1913. Condition has been good. Received two tubes of culture. Jan. 23. Re-

ceived two tubes of culture. Jan. 29. Received two tubes after passage through seven mice. Feb. 19. Received two tubes. Feb. 24. Limps slightly with left fore leg. Feb. 25. Received two tubes of human blood agar culture. March 1. Received two tubes of human blood agar culture. March 13. The same. March 21. The same. March 24. Suffers from scabies. Heart sounds normal, but animal weak. Etherized.

*Autopsy.*—A few whitish streaks occur in the endocardium of the left ventricle, and the epicardium is slightly opaque. The organs are normal except that the spleen shows a few minute scars in the capsule.

*Microscopical Examination.*—Stained with hematoxylin and eosin. Sections of the heart show throughout numerous focalized lesions of round cell infiltration and fibroblastic proliferation with slight atrophy of muscle fibers corresponding typically with those of Bracht and Wächter. Aschoff bodies are absent. There occurs also a slight diffuse round cell infiltration of the myocardium. Methyl-green pyronin stain shows the round cells within the lesions to consist mainly of large mononuclears and to a small extent of small mononuclear and polymorphonuclear cells.

*Rabbit 15.*—White. Injected with organism 211 (epidemic streptococcus). Dec. 18, 1912. Received one culture on serum glucose agar. Dec. 19. Left hind leg spared. Dec. 20. Limping and ill; coughs. Five drops of blood yield 1,000 colonies. Dec. 21. Moribund; chloroformed.

*Autopsy.*—Ten drops of blood from heart when plated gave innumerable colonies. Streptococci in pure culture from urine; bile sterile. Heart: grayish white streaks and dots occur in the right ventricle and on its endocardium; hemorrhages occur in the ventricular endocardium beneath the aortic flap of the mitral valve. Lungs: diffuse bronchopneumonia. The kidneys contain focalized purulent streaks in the cortex, and edema of the pelvis and medulla.

*Microscopical Examination.*—Stained with hematoxylin and eosin. Sections of the wall of the left ventricle show irregular areas, some of which exceed in size the low power field of the microscope, but no areas are fusiform in shape. Within the areas the muscle fibers show various stages of hyaline degeneration, necrosis, and fragmentation, while an edema separates widely the muscle and connective tissue fibers from one another. A moderate leucocytic infiltration, mainly mononuclear and partly polymorphonuclear, is present. Cellular and nuclear debris stain deep blue. In the inter-cellular and lymph spaces a granular, blue staining material composed of masses of streptococci occurs. Elsewhere in the sections there are slight edema and numerous muscle fibers showing vacuolation and hyaline degeneration. Certain capillaries are filled with

mononuclear leucocytes. The methyl-green pyronin stain brings out in addition some plasma cells in the exudate and in the capillaries. The wall of the right ventricle near the septum shows the same condition and a fibroblastic proliferation about some of the areas of degeneration. The Gram-Weigert stain shows masses of streptococci in the neighborhood of the areas of degeneration and small scattered numbers between the muscle fibers.

#### DISCUSSION.

From the above it appears that by inoculating rabbits with cultures of *Streptococcus mitis* obtained from cases of subacute bacterial endocarditis, focalized lesions may arise identical with those produced with *Streptococcus rheumaticus* and by Bracht and Wächter with organisms having similar cultural characteristics to the latter. The lesions are entirely different from the submiliary myocardial rheumatic nodules described by Aschoff. The former have neither the structure, location, type of cell, nor staining reactions of the latter. Aschoff bodies consist of rosette, or fan-shaped, collections of large, irregular, often multinuclear cells with rather dense granular protoplasm and vesicular nuclei, arranged about the adventitia of the medium sized arteries or beneath the endocardium, or of fusiform groups of cells lying between the muscle fibers. The cells possess basophilic protoplasm which stains an intense red with methyl-green pyronin. The experimental lesions, however, differ in every way except in being focalized. The lesions in the myocardium of the rabbit are a combination of degenerative and productive processes. Degeneration of the muscle fibers occurs either accompanied by or soon followed by a proliferation of the interstitial tissue which is disproportionate to the extent of the myocardial change. The Aschoff bodies present the character of a productive process in which a specific type of cells with a particular arrangement and location coexist, and in which the lesion becomes replaced later by fibrous tissue. The lesions described by Jackson and Coombs, while differing from those described by us, differ also from Aschoff bodies, so that further evidence is needed to prove that they are a stage in the development of Aschoff bodies produced experimentally.

We have previously reported finding in the hearts of patients with subacute bacterial endocarditis the same type of lesions as those occurring in our rabbits, which we have called "Bracht and Wächter type of lesions." In several instances the culture inoculated came from these cases. In only two of the hearts were Aschoff bodies found, and in one from a patient giving a history of rheumatism, they were in the healed stage. In the other, a typical verrucose endocarditis and numerous typical Aschoff bodies coexisted. The Aschoff bodies occurred apart from the Bracht and Wächter type of lesions and no relation between them or transition from one to the other was detected.

Rabbit 29 shows that a streptococcus which when given in lethal doses produces pyogenic myocardial lesions, in sublethal doses administered over a long period of time gives rise to a chronic type of focalized lesion similar to that of Bracht and Wächter.

The fact that *Streptococcus mitis* is not found in the lesions unless vegetative endocarditis occurs coincidentally, indicates that if those organisms are directly responsible for the focalized changes either they disappear early or the lesions result not from the organisms themselves directly but from some toxic product. These possibilities have been considered by Bracht and Wächter and de Vecchi. The former think it probable that the attenuated streptococci are easily destroyed in the body. De Vecchi thinks that if *Streptococcus rheumaticus* is the etiological cause, it should be recognized in all cases.

Since we have demonstrated by cultures *Streptococcus mitis* in inflamed joints of rabbits a considerable time after their localization, it would seem that the streptococci are not more perishable than many other bacteria; and hence we incline towards the view that the focalized myocardial lesions are caused by toxins. It is known that diphtheria toxin causes focalized cardiac lesions; and Loeb and Fleischer have produced similar lesions by means of adrenalin and spartein. The fact that there are a number of substances capable of producing focalized lesions in the myocardium shows definitely that the power to cause degenerative and productive myocardial foci is not a specific property of any one substance or bacterium. Therefore the capacity of *Streptococcus rheumati-*

*cus* to produce myocardial focalized lesions can be looked upon simply as a property which this organism shares with other bacteria, bacterial and other substances, and is in no way specific. Since the experimental lesions caused by these agents agree in no way with the Aschoff bodies, except as regards the focalized nature, they can not be used as an argument for the etiological relation of *Streptococcus rheumaticus*, or *Streptococcus mitis (viridans)*, to acute rheumatic fever.

Rosenow claims that the organisms which he has recovered from cases of acute rheumatic fever soon lose in subculture the properties which cause them to produce characteristic lesions in rabbits. The lesions reported above have been produced by various cultures of *Streptococcus mitis*, some recently isolated and others old, and by five strains of *Streptococcus rheumaticus*, all of which were old. We have found the lesions caused by these two types of organisms to be identical. We have not had the opportunity of working with *Streptococcus rheumaticus* in recently isolated cultures.

#### CONCLUSIONS.

1. By the intravenous injection into rabbits of *Streptococcus mitis*, we have produced focalized myocardial lesions which are identical with those caused by the injection of *Streptococcus rheumaticus*, and with those produced by Bracht and Wächter with *Streptococcus viridans*.
2. The lesions differ from those which we produced by injections of streptococci from the Chicago epidemic of sore throat (epidemic streptococcus).
3. The lesions are not identical with Aschoff bodies and are easily differentiated from them. They also differ from the foci produced by Jackson and Coombs, who describe their lesions as being either Aschoff bodies or similar formations.
4. The myocardial lesions of the rabbit appear to be caused by toxins liberated by the streptococci injected and not by the living organisms themselves.
5. The only point of similarity between the experimental lesions and those found in cases of rheumatic carditis in man is their focalized nature.

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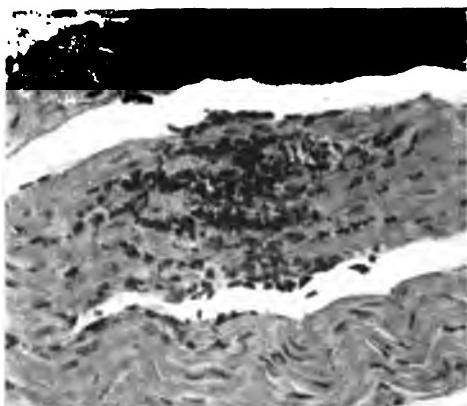


FIG. 1.

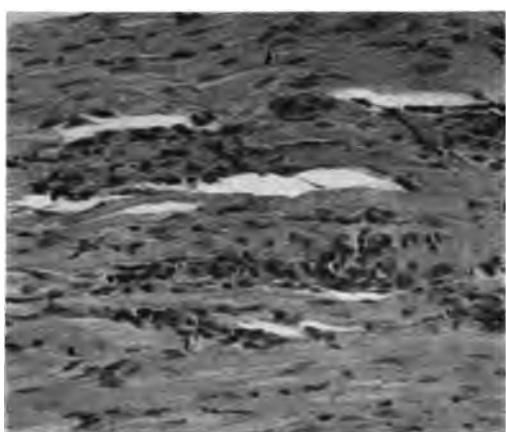


FIG. 2.

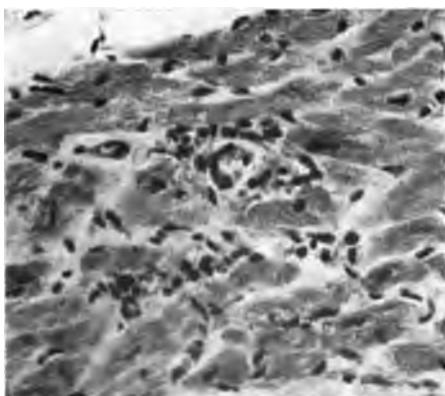


FIG. 3.

(Thalhimer and Rothschild: Focalized Myocardial Lesions.)



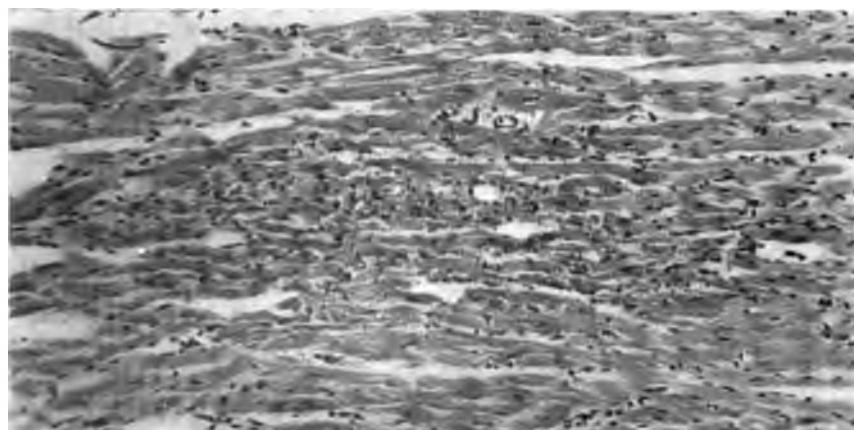


FIG. 4.

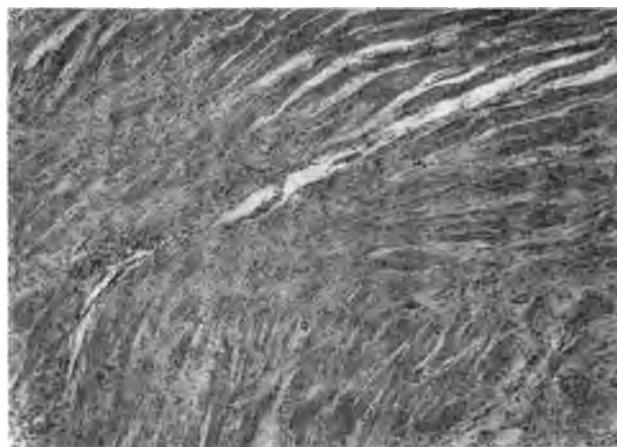


FIG. 5.

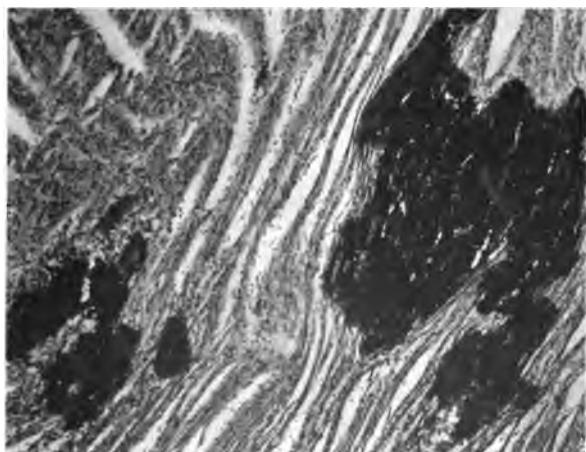


FIG. 6.

(Thalhimer and Rothschild: Focalized Myocardial Lesions.)



## EXPLANATION OF PLATES.

## PLATE 49.

FIGS. 1 and 2. Earliest stage of focalized myocardial lesion with leucocytic infiltration and beginning degeneration of muscle fibers. Hematoxylin and eosin.  $\times 112$ .

FIG. 3. Perivascular infiltration, early stage. Hematoxylin and eosin.  $\times 144$ .

## PLATE 50.

FIG. 4. Early stage of focalized myocardial lesion, but more advanced than in figures 1 and 2, showing leucocytic infiltration, degeneration, and fragmentation of muscle fibers and beginning fibroblastic proliferation. Hematoxylin and eosin.  $\times 144$ .

FIG. 5. Late stage of myocardial lesion showing marked fibrosis of a diffuse type, with here and there leucocytic infiltration. Hematoxylin and eosin.  $\times 48$ .

FIG. 6. Myocardial lesion showing calcareous infiltration. Hematoxylin and eosin.  $\times 144$ .

## EXPERIMENTAL ARTHRITIS IN THE RABBIT, PRODUCED WITH STREPTOCOCCUS MITIS.\*

By M. A. ROTHSCHILD, M.D., AND WILLIAM THALHIMER, M.D.

(From the Pathological Laboratory of the Mount Sinai Hospital, New York.)

Arthritis has been produced in animals by means of various microorganisms. Those persons who advocate the specificity of *Micrococcus rheumaticus* in acute rheumatic fever find much positive support in the results obtained from the inoculation of animals in which arthritis, endocarditis, or pericarditis occur. In view of this consideration we have been led to inject rabbits with *Streptococcus mitis* isolated *intra vitam* from cases of subacute bacterial endocarditis, with the results to be described in this paper.

This communication does not deal with the etiology of rheumatism. Clinicians and pathologists must first decide what shall be termed acute articular rheumatism. However, as we<sup>1</sup> have pointed out, one group of cases of rheumatism exists in which there is a characteristic myocardial lesion (Aschoff bodies) associated with a verrucous endocarditis and unattended by embolic phenomena. Bacteriological examination in these cases is negative. There is a large second group comprising the bacterial infections of the endocardium, and a third group of bacterial or toxic arthritides with or without endocarditis.

It is possible to produce arthritis in rabbits by means of *Bacillus pyogenes fetidus liquefaciens*, staphylococci, hemolytic streptococci, the streptococcus of epidemic sore throat, Achalme's bacillus, *Micrococcus rheumaticus* isolated from the throat, blood, and joints, *Streptococcus salivarius* and *fecalis* (Horder), the pneumococcus, and *Streptococcus viridans*. Rosenow has reported the production of arthritis with *Streptococcus viridans* in a small percentage, and of endocarditis in a large percentage of rabbits. Our figures have been quite the reverse; that is, 50 per cent. of our rabbits developed

\* This work was done under the tenure of a George Blumenthal, Jr., Fellowship. Received for publication, February 3, 1914.

<sup>1</sup> Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 417.

arthritis and 7 per cent. endocarditis. We have employed nine different strains of *Streptococcus mitis* (table I). By reference to the table it will be seen that there is a slight variation in the ability of the different strains to produce arthritis.

TABLE I.

Organism.	No. of rabbits.	Arthritis.	Endocarditis.
4.110	10	7	2
4.147	9	3	0
4.206	7	3	1
4.275	6	5	0
4.170	3	2	0
4.342	3	1	0
4.357	2	1	0
2.524	1	0	0
C	1	0	0
	42	22	3
		50%	7%

## CHARACTER OF THE ARTHRITIS.

The rabbits described here are the same as those reported in our communication<sup>2</sup> in which the method of injection, the media used,

TABLE II.<sup>3</sup>

Rabbit 53. Organism 4.275. Injected May 26, 1913. 5 Injections.

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
June 9.....	+	-	+	+
June 14.....	++	-	++	++
June 16.....	++	-	+	++
June 24.....	+++	-	-	+
June 29.....	+++	-	-	+
July 3.....	+++	+	-	+
(Elbow)				
	+			
(Wrist)				
July 7.....	+	-	+	+
July 9.....	+	-	+	+
July 21.....	++	-	-	++
July 28.....	++	-	+	++
Aug. 25.....	-	+	+	+
Sept. 3.....	+	+	-	+
Killed				

<sup>2</sup> Thalhimer, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 429.

<sup>3</sup> Beattie (*Jour. Path. and Bacteriol.*, 1910, xiv, 436, table VI) shows in a graphic manner the involvement of the joints of a rabbit that was injected with a streptococcus resembling *Streptococcus rheumaticus*. His table corresponds closely to tables II, III, IV, and V in our series.

\* In the tables, + = joint affected; — = joint not affected.

and the doses are given. The production of arthritis appears to be independent of the doses of the streptococci and the media employed.

By reference to tables II, III, IV, and V the evanescent and mi-

TABLE III.

*Rabbit 10. Organism 4,147. Injected December 10, 1912. 6 Injections. Killed January 24, 1913.*

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
Dec. 26.....	—	—	—	Limps; ankle swollen.
Dec. 27.....	—	—	—	No limp; ankle swollen.
Dec. 28.....	—	—	—	—
Dec. 29.....	—	—	—	Ankle swollen.
Dec. 31.....	—	—	—	Ankle swollen.
Jan. 6.....	—	—	—	Ankle swollen.
Jan. 13.....	—	—	—	Swelling smaller.
Jan. 17.....	—	—	—	Swelling smaller.
Jan. 22.....	—	—	—	Swelling smaller.
Jan. 24.....	—	—	—	Swelling smaller.

TABLE IV.

*Rabbit 18. Organism 4,110. Injected December 22, 1912. 4 Injections. Died February 20, 1913.*

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
Dec. 25.....	—	—	Limps	—
Dec. 27.....	—	—	Ankle swollen	—
Dec. 31.....	—	Stiff	Stiff	—
Jan. 13.....	—	—	—	—
Jan. 18.....	Limps; ankle swollen	—	—	—
Jan. 25.....	—	—	—	—

TABLE V.

*Rabbit 70. Organism 4,357. Injected June 24, 1913. 2 Injections. Died July 13, 1913.*

Date.	Left fore foot.	Right fore foot.	Left hind foot.	Right hind foot.
June 27.....	—	—	Stiff	Stiff
June 28.....	—	—	+	+
June 29.....	—	—	+	+
July 3.....	—	—	Knee +	Knee +
July 7.....	—	—	+	++
July 9.....	—	—	+	+
July 13.....	—	—	—	—

gratory character of the arthritis is seen. Authors have used these characters as an argument for the specificity of a special organism

in causing acute articular rheumatism. The fact that the joints after extensive involvement return to a normal condition has been emphasized by Poynton and Paine, Beattie, and others. The arthritis produced by *Streptococcus mitis* has the same general characteristics. In some of the animals a chronic type of arthritis arises, the joint cartilages being eroded, the articular surfaces proliferated, and a partial ankylosis of the joint resulting. Poynton and Paine have seen the same results follow from *Micrococcus rheumaticus*. For control experiments we injected a number of rabbits with streptococci obtained from the sore throat epidemic in Chicago. Our results agreed with those of Davis who produced arthritis in every animal inoculated with organisms from the Chicago and Boston epidemics of sore throat. We injected a series of rabbits with hemolytic streptococci, and our results agreed with those of Cole who states: "In most cases the pathological changes in the joints are mild, consisting mainly of edema about the joint, the fluid becoming turbid, sticky, and tenacious, injection of the capsule and slight infiltration, mainly of the villi, with polymorphonuclear leucocytes." The arthritides which developed in our series were acute in their development, almost purulent in nature, and accompanied by some destruction of the surfaces of the articular cartilages. A series of rabbits (eleven) was injected separately with five different strains of *Streptococcus rheumaticus*, one strain isolated by Poynton and Paine, one by Beattie, one by Lintz, and two by Rosenow. 45 per cent. of the animals developed an arthritis; and the character of the arthritis was identical with that caused by *Streptococcus mitis*.

#### CHARACTER OF THE JOINT EXUDATE.

The exudate was of the same character as that described by the observers working with *Streptococcus rheumaticus*. It was of a thick, sticky, gelatinous consistence and contained many large endothelial cells with distinct phagocytic properties, a moderate number of polymorphonuclear leucocytes, and a large quantity of homogeneous material. The exudate is both articular and periarticular, having a decided tendency to extend down the tendon sheaths, and in one rabbit it extended from the knee joint to the ankle. In only

one instance was there any tendency towards decided pus formation.

Bacteriological studies of the exudate (table VI) show that we were able to demonstrate microorganisms in a comparatively small

TABLE VI.

Organism.	No. of rabbit.	Blood cultures.	Joints.	
			Smears.	Cultures.
4.147	1	o	o	+
4.147	10	o	o	o
4.142	43	o	o	+
4.170	27	Not taken	o	o
4.170	43	o	o	o
4.357	70	o	o	o
4.110	18	o	o	o
4.110	36	+	+	+
4.110	37	o	o	o
4.110	38	+	o	o
4.110	47	o	o	o
4.110	48	o	+	o
4.110	49	Autopsy records lost		o
4.206	39	o	+	o
4.206	57	o	o	o
4.206	59	+	+	+
4.275	35	o	+	+
4.275	41	o	o	o
4.275	51	o	o	o
4.275	52	o	o	o
4.275	53	o	+	o
4.342	55	o	+	o

percentage. In smears the organisms are almost always found intracellularly both in the large endothelial cells and in the polymorphonuclear leucocytes. The organisms can be recovered in cultures in only about one third of the animals. In one rabbit with a positive blood culture both smears and cultures from the joint were negative. Two of the rabbits having a vegetative endocarditis with positive blood cultures gave cultures from the joints. In the table bacteriological studies of the joints which have subsequently returned to normal are included; consequently the figures may not give a true index of the relative finding of streptococci in the affected joints. Meyer claims as one of the characteristics of the arthritis produced by *Streptococcus rheumaticus* that organisms can not be recovered from the joints. Other observers, however, have isolated the organism in the joints of cases of rheumatism and in arthritis produced experimentally in animals.

## CONCLUSIONS.

1. We have produced arthritis in 50 per cent. of the rabbits injected with *Streptococcus mitis*.
2. The character of the arthritis is identical with that produced by *Micrococcus rheumaticus*.
3. The exudate in and about the joints partakes of the same nature as that caused by *Streptococcus rheumaticus*.
4. Bacteriological studies show that *Streptococcus mitis* can be recovered from about one third of the affected joints.
5. Arthritis produced by other types of streptococci differs by reason of greater destruction of tissue, by being more permanent in character, and by the exudate containing large numbers of polymorphonuclear leucocytes.
6. The deduction of a distinct variety or species of streptococcus based upon the power to cause arthritis in rabbits is unwarranted.

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A STAGE IN THE MIGRATION OF THE ADULT TERTIAN MALARIAL PARASITE. EVIDENCE OF THE EXTRACELLULAR RELATION OF THE PARASITE TO THE RED CORPUSCLE.\*

By MARY ROWLEY-LAWSON, M.D.

(*From the Laboratory of Dr. Mary Rowley-Lawson, New London.*)

PLATES 51 TO 55.

In previous publications<sup>1</sup> I have stated that I believe the malarial parasite to be extracellular and to migrate from corpuscle to corpuscle. To recapitulate:

I believe the malarial parasite to be extracellular throughout its existence; that is, except for brief periods when it is free in the blood serum, it is attached to the external surface of the red corpuscle.

The parasite attaches itself to the red corpuscle by means of delicate pseudopodia thrown out from the cytoplasm of the parasite. It encircles and squeezes up into a mound a portion of the hemoglobin (figures 1 to 17); thus it maintains its position on the outside of the red corpuscle.

From my observations I believe that each parasite in the course of its development, destroys several red corpuscles, migrating from one to another, thus giving a rational explanation of the anemia which occurs in the course of the malarial infections.

The evidence in favor of migration is as follows: 1. The great destruction of red corpuscles, which is usually out of all proportion to the number of parasites present in the peripheral blood, provided that each parasite destroys but one corpuscle. 2. Instances of multiple infection of red corpuscles by several young parasites,

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<sup>1</sup> Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420; *Jour. Exper. Med.*, 1913, xvii, 324.

sometimes as many as seven. They cannot all grow on one corpuscle, and even if conjugation were amongst the possibilities, it would not account for odd numbers nor for infection of a corpuscle by parasites of varying stages of development. Therefore if these parasites are not to die, they must migrate. 3. What appear to be stages in parasitic migrations, which may be summarized as follows :  
(a) Pigmented parasites free in the blood serum. They may be seen in various stages of development, compact, ring-shaped, ameboid, and with protoplasmic pseudopodia, the pigmentation being evidence of previous attachments to red corpuscles. (b) Pigmented parasites in various stages of development, attached to red corpuscles the hemoglobin of which is apparently unaltered. Here, as in stage (a), the pigmentation is evidence of one or more previous attachments. (c) Pigmented parasites in various stages of development on decolorized red corpuscles and on corpuscular skeletons. (d) Pigmented parasites partly on and partly off degenerated red corpuscles, caught apparently in the act of abandoning them. (e) Corpuscular skeletons, which are the expanded, dehemoglobinized remains of red corpuscles, usually more or less semilunar in shape, granular, and staining a delicate pink. These skeletons are most frequently seen free from parasites.

This paper is intended principally as a description of stage (d), but it illustrates also the extracellular relation of the adult tertian parasite to the red corpuscle. In one specimen of blood from a series of smears taken consecutively, one after another, from a patient who had taken no quinine, I found over 100 thirty (?) hour parasites partly on and partly off expanded red corpuscles showing granular degeneration (figures 19 to 54, and 59 to 78). I have occasionally seen parasites partly off red corpuscles in other specimens; but never so many in any one smear as in this case. Free parasites, in the same stage of development as the parasites seen partly off the red corpuscles, were found in the same smear (figure 55). I believe that these parasites were preparing to migrate, for the infected red corpuscles, though retaining in general their contour, were undoubtedly degenerated,<sup>2</sup> being more or less dehemo-

<sup>2</sup> Segmenting parasites on red corpuscles showing similar granular degeneration (figures 56 to 58) were found in the same smear.

globinized. Finding so many parasites in similar stages of development in this phase, in one smear, seems to me good evidence in favor of migration as against a condition caused by technique. I can think of no evidence in favor of technique that may be responsible for the phenomenon. The smear was made on a slide, with the blood spread only in one direction, yet the parasites were seen reaching away from the corpuscles in various directions. If rough smearing was the cause of the parasites being partly off the corpuscles, the parasites would be drawn off from the corpuscles all in one direction. And technique would not account for a condition where the corpuscle is distorted exactly at right angles to the direction in which the parasite reaches out from the corpuscle (figures 22 and 27).

In many instances that portion of the parasite which is off the red corpuscle, may be seen resting along the periphery of the infected cell (figures 20, 31, 32, 34 to 37, and 39), which would not be the case were technique responsible for its dislocation.

That the adult parasite is attached to the external surface of the red corpuscle and not submerged beneath its surface seems to me to be proved by figures 1 to 58.

Parasites developed beyond the young ring-form stage (figures 1 to 11) may occasionally be seen to be attached to fairly healthy appearing red corpuscles, the bodies of the parasites resting on the periphery of the corpuscles (figures 13 to 16). These parasites are certainly not submerged beneath the surface of the red corpuscles, but have, I believe, only recently attached themselves, and are therefore coming rather than going. Occasionally a segmenting body may be seen extending beyond the periphery of a red corpuscle with unbroken contour (figure 18).

As to the parasites that are preparing to migrate (figures 19 to 54), to the trained eye those that are on the upper surface of the granular degenerated corpuscles can easily be differentiated from the parasites on the under surface; i. e., in figure 22, the parasite is partly off the upper surface of the red corpuscle, while in figure 33 the parasite is seen to be partly off the under surface of the corpuscle.

The unbroken contour of the majority of the infected red cor-

puscles showing portions of parasites extending beyond the periphery, as well as appearance of the adjacent cells, with the exception of a in figure 36, would seem to preclude any suggestion of unusual violence in the spreading of the smear. I use the adjective unusual, as it seems to me that it would require a very unusual violence to produce a condition as rare as that seen in the accompanying plates. I believe the infected red corpuscles were already too much damaged by the action of the parasites to regain their contour on the slide if the parasites had been forcibly squeezed or pulled out of them in spreading the smear, especially parasites of such advanced growth. I do not believe that in smearing the blood it would ever be possible to reach the parasite, if it were submerged beneath the surface of the red corpuscle, in order to pull or squeeze it out, without damaging that corpuscle beyond repair; for instance, in figure 20 the body of the parasite rests on the periphery of the corpuscle and only short pseudopodia are seen to be attached to the corpuscle, the contour of which is unbroken; and in figure 19 only a slender process extends beyond the periphery of a corpuscle with unbroken contour. If these parasites were submerged beneath the surface of the red corpuscles, could technique have produced the condition? I think not.

Healthy appearing red corpuscles may be damaged by technique when the blood is being smeared and not regain their contour. The healthy, uninfected, but distorted, red corpuscle seen at a in figure 35 did not regain its shape after being injured by obvious technique. In malarial infections, especially in the æstivo-autumnal infections I have seen many red corpuscles which had not regained their normal contour after having been damaged by the parasites while in the circulating blood.<sup>8</sup>

If the young parasite fastens itself to the external surface of a red corpuscle and proceeds with the destruction of the corpuscle while it is so attached (see young parasites encircling corporcular mounds on the decolorized red corpuscles in figures 10 and 11), why should the adult parasite follow any other procedure? Indeed, peripheral mounds may be seen in connection with adult parasites

<sup>8</sup> An article describing and illustrating these damaged red corpuscles will appear later.

(figure 17, a). And why should the adult parasite assume a more or less characteristic ring-form unless it be for the purpose of securing its attachment to the surface of the red corpuscle? For the ring shape, in connection with the adult parasite, may be explained, as it is explained in connection with the young parasite, as a result of the parasite encircling and drawing up into a mound a portion of the hemoglobin substance of the red corpuscle in order to secure its attachment to the surface of the corpuscle.

In fresh blood preparations, parasites may be seen to abandon red corpuscles which have not been entirely destroyed. Laveran,<sup>4</sup> in referring to the external relation of the malarial parasite to the red corpuscle, says: "Osler noticed that the amoeboid bodies which adhere to the red blood corpuscles can be detached and become free in the blood," adding, that this was one of the arguments on which he relied in maintaining that the parasites were only attached to the surface of the red corpuscles. Marchiafava and Bignami<sup>5</sup> and Monacho and Panichi<sup>6</sup> have stated that quinine may cause certain parasites to abandon the red corpuscles. This being so, if the parasites were submerged beneath the surface of the red corpuscles, then the quinine would have to destroy the substance of the red corpuscles in order to release the parasites; but with the parasites attached to the external surface of the corpuscles, their detachment is more easily explained.

#### SUMMARY.

1. What appear to be certain definite stages in the migration of the malarial parasite from red corpuscle to red corpuscle may be demonstrated by thorough and persistent observations,—not minutes spent on each specimen, but many hours.

2. The migration of the malarial parasite from red corpuscle to red corpuscle gives a reasonable explanation of the loss of red corpuscles which cannot be accounted for by the destruction of the infected corpuscles at the time the parasites segment.

3. Migration to other red corpuscles is a satisfactory explana-

<sup>4</sup> Laveran, A., *Paludism*, translated by Martin, J. W., London, 1893, 41.

<sup>5</sup> Marchiafava, E., and Bignami, A., in *Twentieth Century Practice of Medicine*, New York, 1900, xix, 461.

<sup>6</sup> Monacho and Panichi, cited by Ewing, J., *Clinical Pathology of the Blood*, 2d edition, New York and Philadelphia, 1903, 458.

tion of the ultimate fate of the young parasites seen in instances of multiple infection of single corpuscles.

4. In the light of the facts here presented, it would seem impossible to explain the instances of the parasites partly on and partly off degenerated red corpuscles as the result of technique. A stage in the migration of the parasite seems to me to be the probable interpretation of the phenomenon.

5. A corpuscular mound encircled by an adult parasite, when seen at the periphery of the red corpuscle, should have the same significance and interpretation in reference to the extracellular relation of the parasite to the corpuscle, that it has when it is seen in connection with a young parasite.

6. The adult ring-form parasite should have the same interpretation as the young ring-form parasite.

7. Attachment to the external surface of the red corpuscles seems to me to be the only possible interpretation of the appearances of the parasites pictured in this article.

#### EXPLANATION OF PLATES.

##### PLATE 51.

###### TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,750$ .

Figs. 1 to 3. Young parasites attached to the external surface of the red corpuscles. Those attached to peripheral mounds may be seen at a; the other parasites are attached to surface mounds.

FIG. 4. The nucleus of the young parasite attached to the peripheral corpuscular mound at a is distorted by technique.

FIG. 5. A young parasite attached to a peripheral corpuscular mound at a, which is almost entirely dehemoglobinized.

FIG. 6. A young parasite attached to the red corpuscle. The body of the parasite rests on the periphery of the corpuscle.

FIG. 7. A young parasite attached to the periphery of a peripheral corpuscular mound. The mound is almost entirely dehemoglobinized.

Figs. 8 and 9. Young parasites encircling peripheral corpuscular mounds.

FIG. 10. A young parasite encircling a surface corpuscular mound; the entire corpuscle is much dehemoglobinized.

FIG. 11. A young parasite encircling a peripheral corpuscular mound. The corpuscle is much dehemoglobinized.

FIG. 12. A pigmented parasite attached to the surface of a red corpuscle. The mounds to which it is attached can be seen at a.

FIG. 13. A pigmented parasite attached to the surface of a red corpuscle, the hemoglobin of which appears to be unaltered. As a large part of the parasite

rests on the periphery of this corpuscle, I believe that the parasite has only recently attached itself to it, hence the unaltered appearance of the hemoglobin.

FIG. 14. A parasite attached to the external surface of the corpuscle. The body of the parasite is seen to be off the corpuscle, having been dislocated by technique.

FIG. 15. A pigmented parasite on the periphery of the red corpuscle, the appearance of the pseudopodia suggesting that the parasite is starting to encircle a corpuscular mound. The slightly degenerated appearance of the infected corpuscle is probably due to the action of the young parasite.

FIG. 16. A pigmented parasite, the body of which rests on the periphery of the corpuscle. This parasite is attached to the under surface of the red corpuscle. There is another parasite attached to the upper surface.

FIG. 17. An adult pigmented parasite attached to the external surface of the red corpuscle. The peripheral mound to which it is attached can be seen at a.

FIG. 18. Young parasites resulting from a very recent segmentation; the red corpuscle has not been entirely destroyed. One of the segments can be seen to be partly off, and two entirely off the corpuscle at a.

FIG. 19. An adult pigmented parasite on the upper surface of a granular degenerated red corpuscle. A pigmented protoplasmic process arising from the parasite can be seen extending beyond the periphery of the corpuscle at a.

FIG. 20. An adult parasite resting on the periphery of a granular degenerated red corpuscle. With the exception of three short and delicate pseudopodia, the parasite is free from attachment to the corpuscle.

FIG. 21. An adult parasite on the upper surface of a degenerated red corpuscle, with a portion of its protoplasm extending beyond the periphery of the infected corpuscle.

#### PLATE 52.

##### TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,750$ .

FIG. 22. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. Note that the parasite is lying off the corpuscle at right angles to the way the cell is distorted.

FIG. 23. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. The mound to which the parasite is attached shows less of the granular degeneration than the rest of the corpuscle.

FIG. 24. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. The surface mound to which the parasite is attached is almost entirely decolorized by the action of the parasite. This mound can be seen at a.

Figs. 25 and 26. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. The mound to which the parasite in figure 26 is attached can be seen at a.

FIG. 27. An adult parasite partly off the under surface of a degenerated red corpuscle. The granular outline of the infected corpuscle can be traced across the upper surface of the parasite between o and o. The mound to which the parasite is attached can be seen at a. Note that the distortion of the corpuscle is at right angles to the way the parasite lies off the corpuscle.

Figs. 28, 29, and 30. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. The mounds of attachment of the parasite in figure 30 can be seen at a.

Figs. 31, 32, and 33. Adult parasites partly on and partly off the under surfaces of degenerated red corpuscles. In figures 31 and 32, the part of the parasite that is off the corpuscle can be seen resting on the periphery of the infected corpuscles.

PLATE 53.

TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,750$ .

FIG. 34. An adult parasite partly on and partly off a degenerated red corpuscle; the part of the parasite that is off the corpuscle may be seen resting on the periphery of the corpuscle.

FIG. 35. An adult parasite partly on and partly off the upper surface of a red corpuscle which is less dehemoglobinized than the other corpuscles shown which are infected by parasites partly off and partly on. At a is a red corpuscle that has been injured by technique and has not regained its normal shape.

Figs. 36 to 39. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. In figures 36, 37, and 39 the part of the parasite which is off the corpuscles can be seen resting on the periphery of the infected corpuscles. In figure 37 a young parasite is seen attached to the infected corpuscle.

FIG. 40. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle. Another adult parasite can be seen on the under surface of the infected corpuscle.

Figs. 41 and 42. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 43. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle. The mound of attachment is seen at a.

Figs. 44 and 45. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. In figure 44 a young parasite is seen attached to the infected corpuscle. The mound to which the adult parasite is attached can be seen at a.

PLATE 54.

TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,750$ .

FIG. 46. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle.

Figs. 47, 48, and 49. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 50. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle.

Figs. 51, 52, 53, and 54. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 55. An example of the free parasites found in the same smear with, and in the same stage of development as, the parasites seen partly on and partly off the degenerated red corpuscles.

Figs. 56, 57, and 58. Segmenting parasites on corpuscles which are in a similar stage of degeneration to many of the corpuscles which show the parasites partly off them, and which came from the same smear.

## PLATE 55.

## TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,750$ .

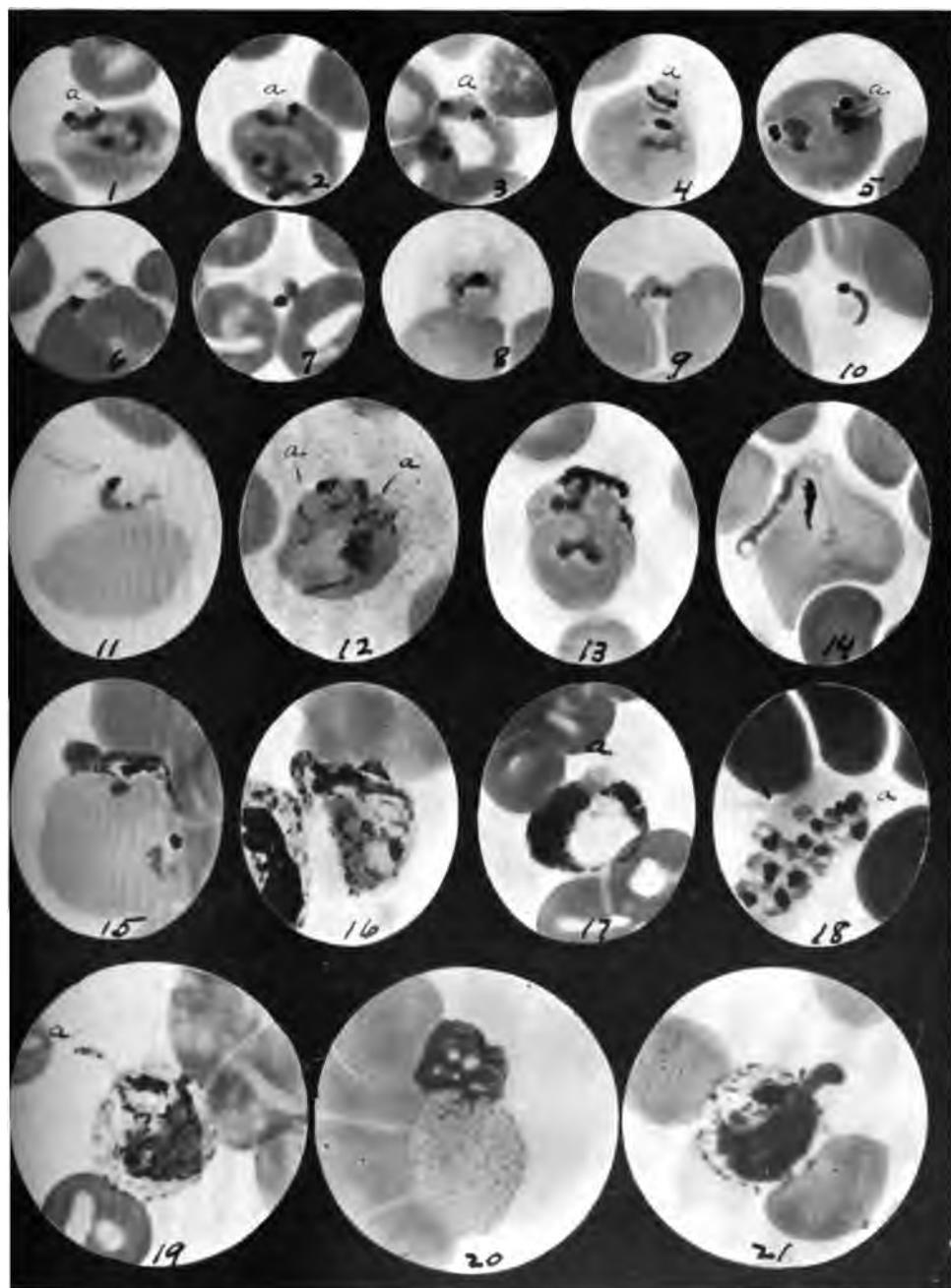
FIG. 59. (Corresponds to figure 18.) A segmenting parasite showing some of the separate segments beyond the periphery of the infected red corpuscle.

FIG. 60. Two young parasites attached to the surface of a red corpuscle; the body of one of the parasites is on the periphery of the corpuscle with the attaching pseudopodium arranged in the form of a loop overlying the corpuscular substance; the other parasite is attached to a decolorized surface mound.

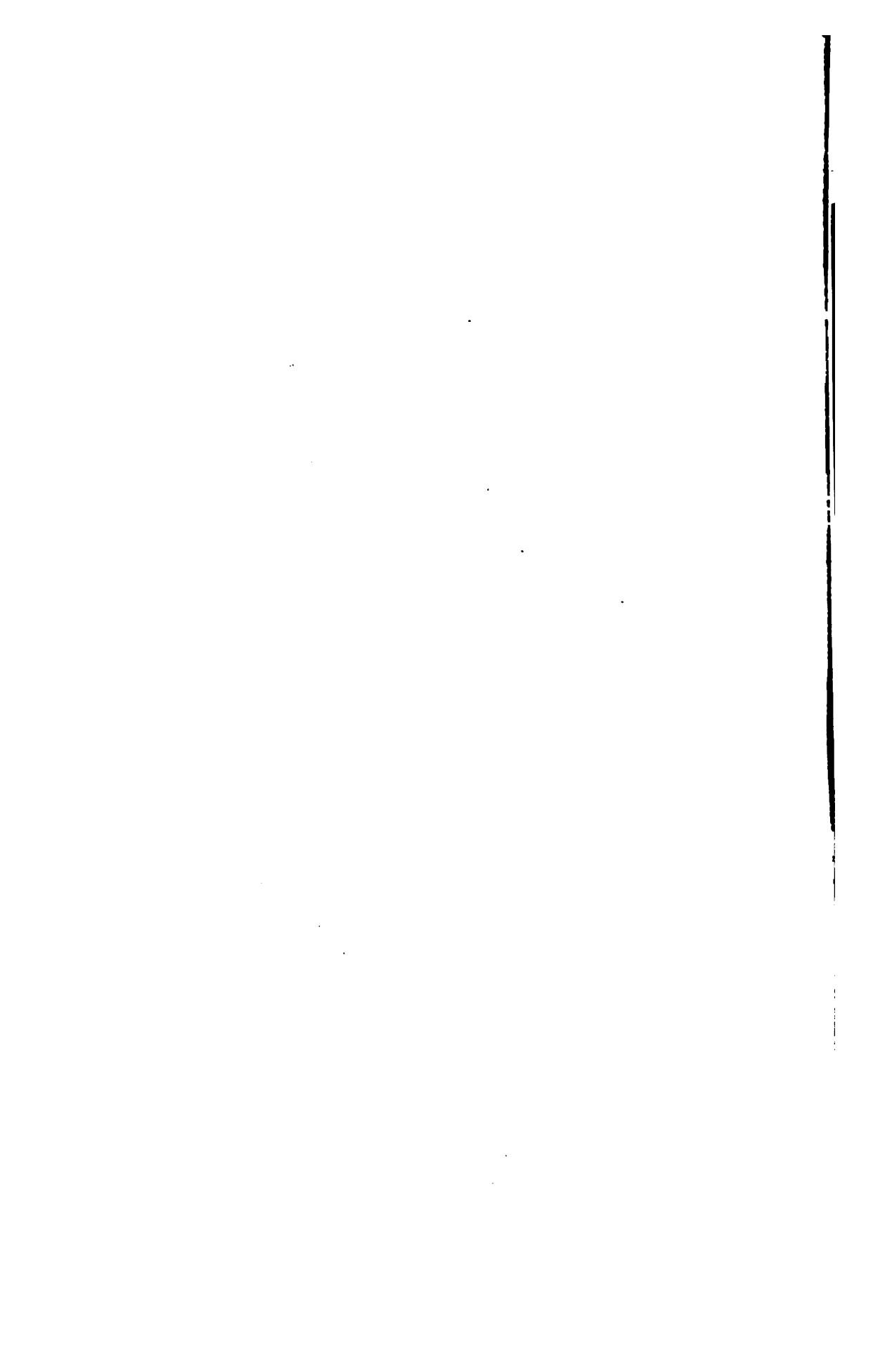
FIG. 61. (Corresponds to figure 12.) A pigmented parasite attached to the surface of a red corpuscle. The mounds to which the parasite is attached can be seen.

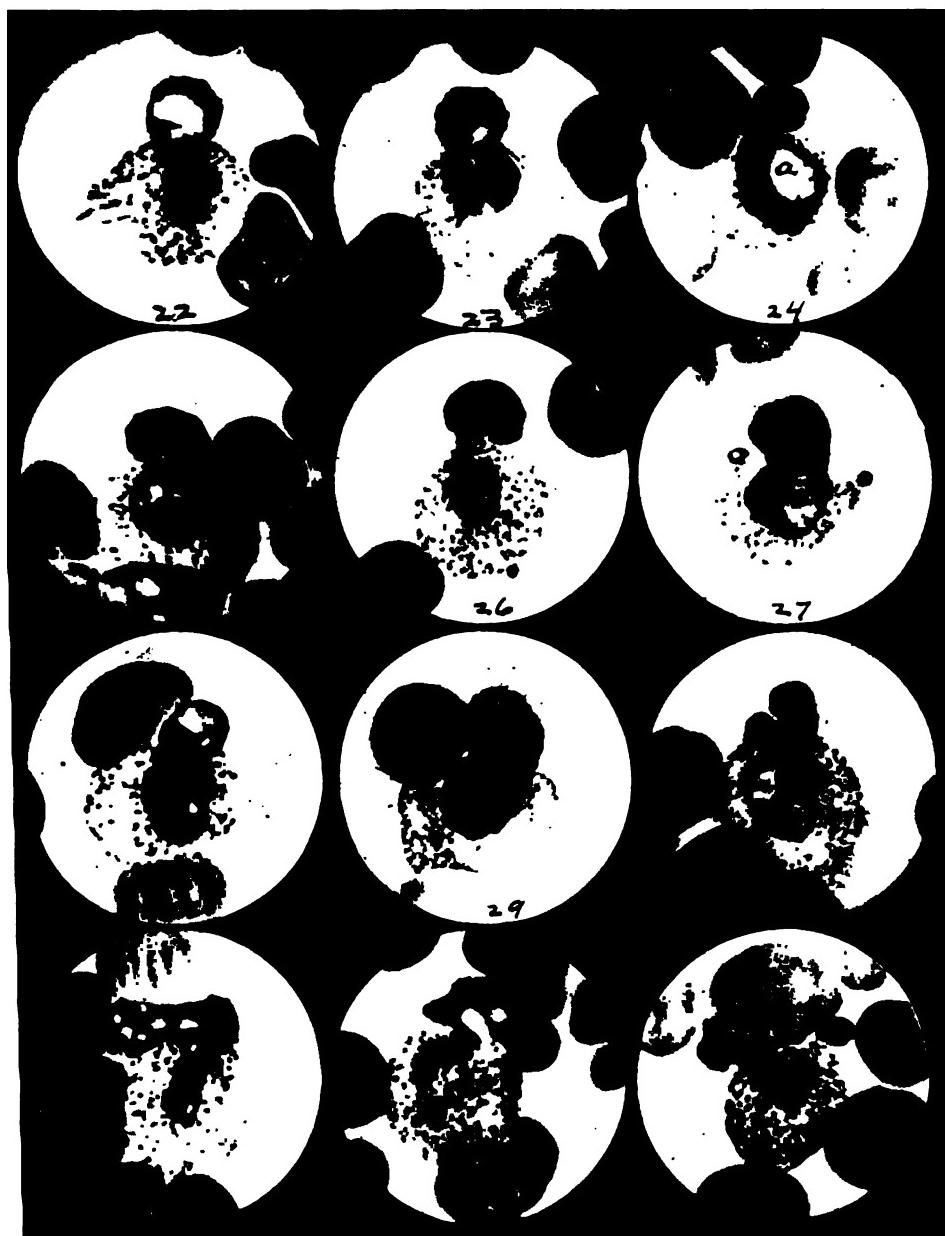
FIG. 62. (Corresponds to figure 15.) A pigmented parasite on the periphery of a red corpuscle, with the pseudopodia overlying the corpuscular substance.

Figs. 63 to 78. (Correspond to figures 25, 28, 23, 24, 37, 26, 30, 22, 29, 33, 32, 31, 21, 20, 19, and 36.) Adult pigmented parasites partly on and partly off granular degenerated red corpuscles. Some of the parasites may be seen to be on the upper surfaces of the corpuscles, others on the under surfaces. In figure 18, with the exception of three short and delicate pseudopodia, the parasite is free from attachment to the corpuscle. In figure 19 only a delicate pseudopodium extends beyond the periphery of a red corpuscle with unbroken contour.

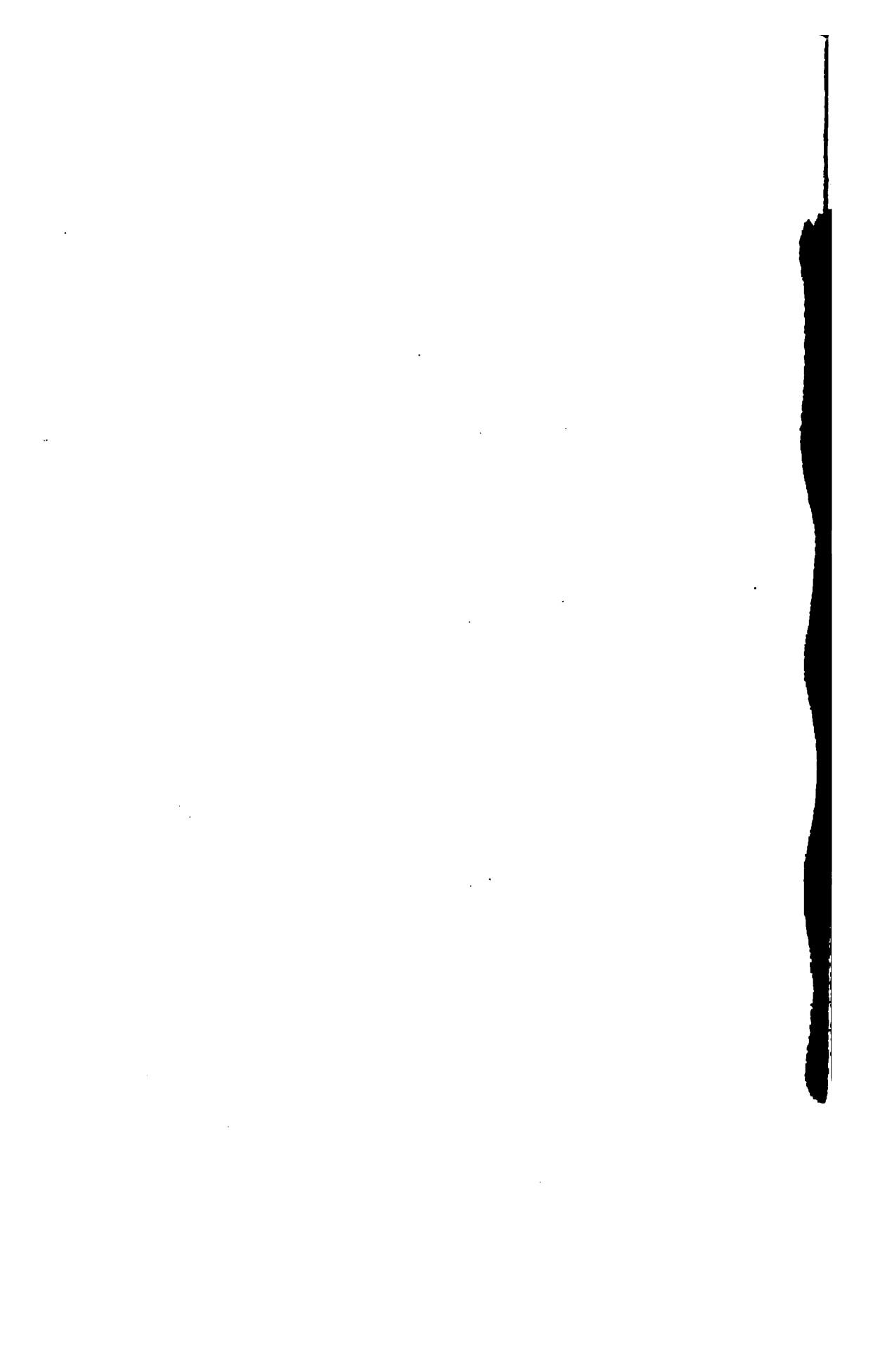


(Lawson: Migration of the Adult Tertian Malarial Parasite.)



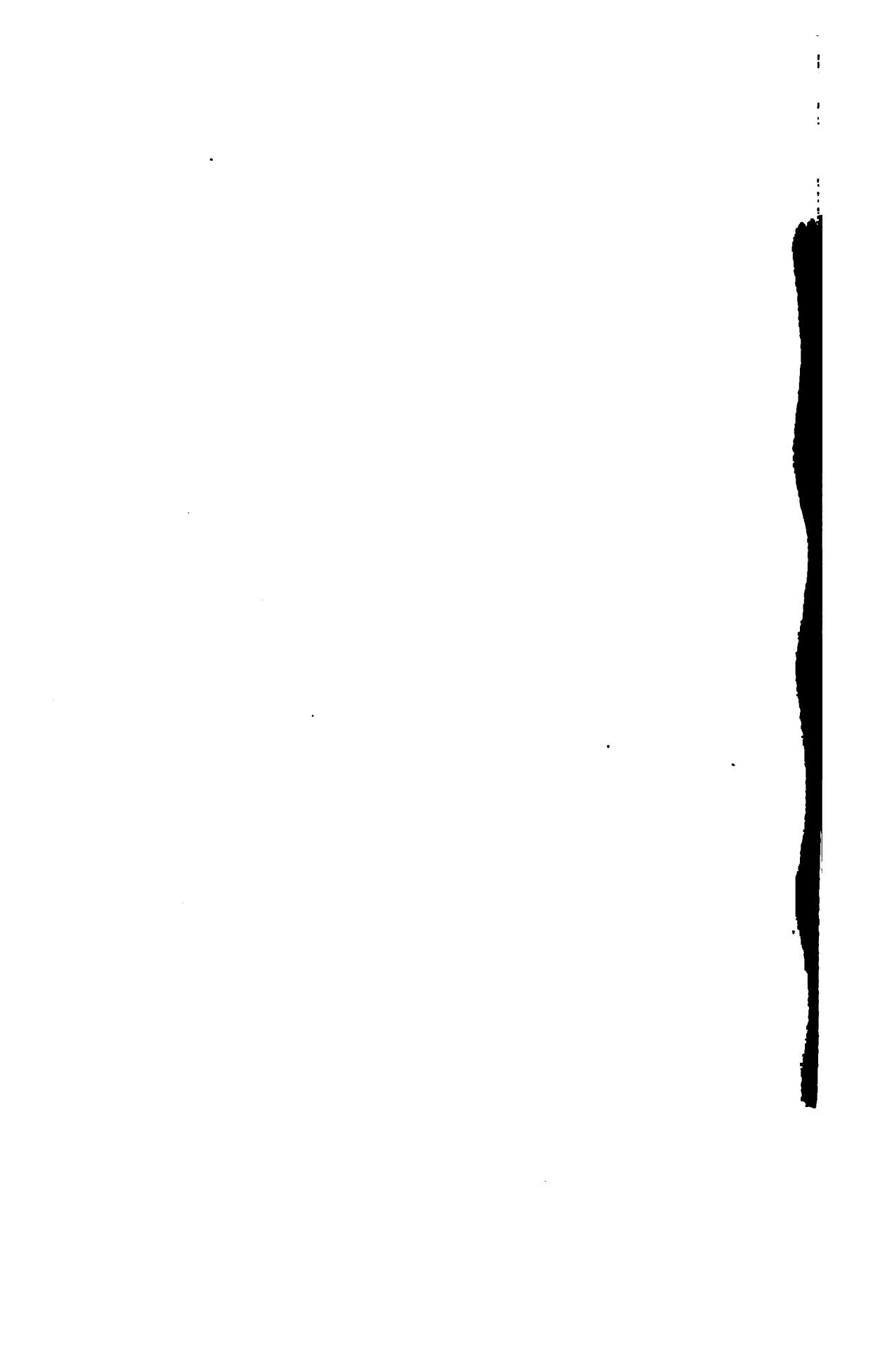


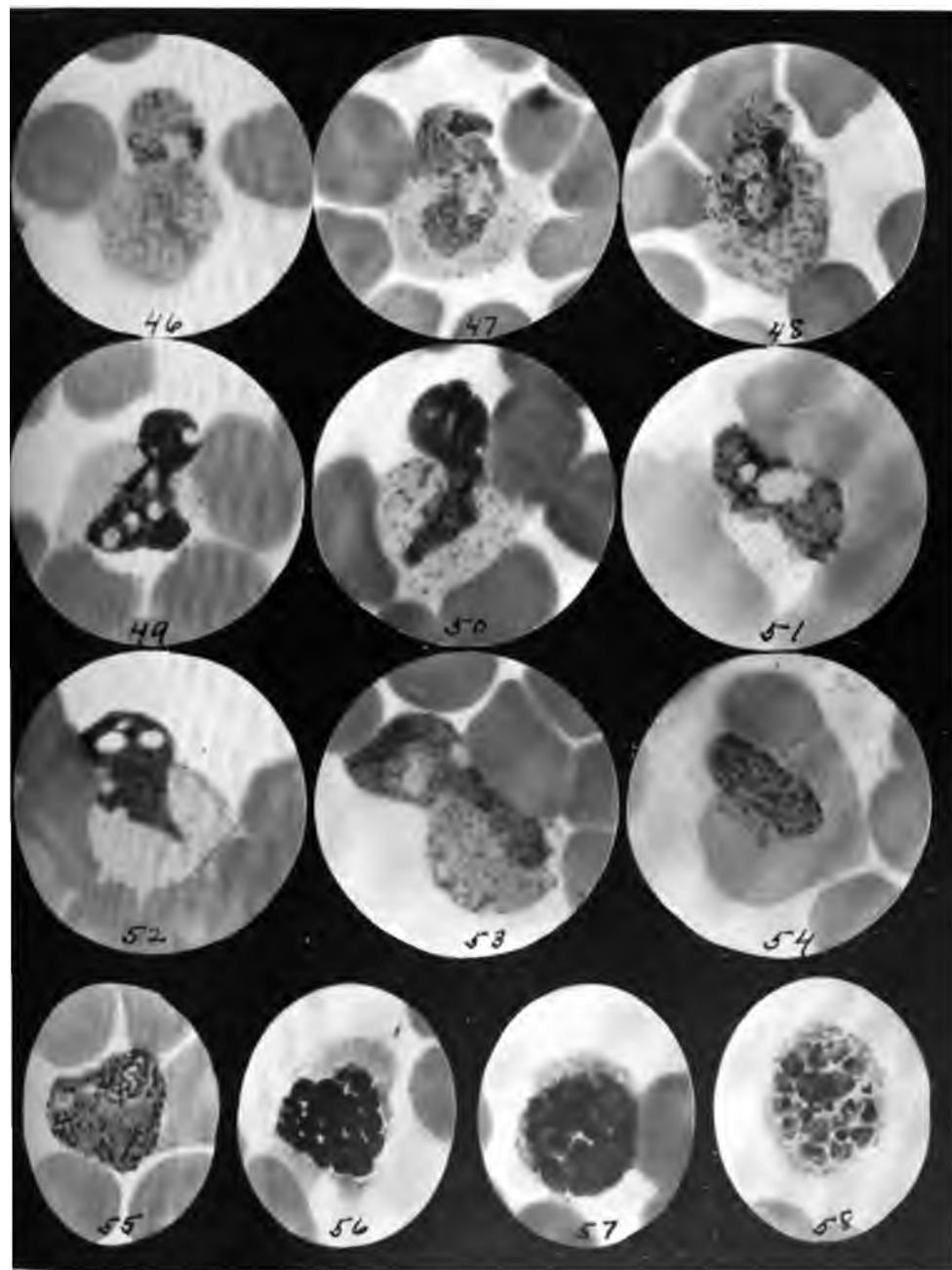
(Lawson: Migration of the Adult Tertian Malarial Parasite.)





(Lawson: Migration of the Adult Tertian Malarial Parasite.) ]





(Lawson: Migration of the Adult Tertian Malarial Parasite.)





(Lawson: Migration of the Adult Tertian Malarial Parasite.)



## THE NATURE OF SERUM ANTITRYPSIN.

### STUDIES ON FERMENT ACTION. XIII.\*

By JAMES W. JOBLING, M.D., AND WILLIAM PETERSEN, M.D.

(From the Department of Pathology of the College of Physicians and Surgeons,  
Columbia University, New York.)

The efforts that have been made to determine the nature of the ferment-inhibiting substances of the blood give no definite information concerning their significance or their character. At one time it was thought that the antitryptic index of the serum would prove to be a valuable aid in the diagnosis of cancer.

Brieger and Trebing (1) stated that 90 per cent. of the patients suffering from carcinoma or sarcoma, whom they had examined, showed an increase of antitrypsin in the blood. Von Bergmann and Meyer (2) confirmed this observation, though they also found a similar increase in 24 per cent. of non-cancerous patients. Recent work indicates that the increased ferment-inhibiting action of the serum cannot be relied upon as a diagnostic test for cancer. It is frequently present in the acute infections, such as pneumonia, typhoid fever, etc.; in chronic infections, such as tuberculosis and syphilis; in Graves' disease; and in severe anemias. The action of the serum has been ascribed to chemical constituents and to specific immune bodies which act as antifermenents. Meyer (3) believes that the antiferment is a true antibody, and that the ferment of the tissue cells act as antigens. He concludes that trypsin and leucoprotease are not so important in this respect. Eisner (4) and Wiens (5) also believe that the antiferment is a true antibody, but that the antigen is the ferment liberated by the polymorphonuclear cells. On the other hand, Jürgensen (6) found no relation between the antitryptic index and leucocytosis. Halpern (7) inoculated dogs with the pancreas of dogs, and found an increase in antitrypsin, but no increase in anti-pepsin. Eisner (4) studied the inhibiting action of serum against various ferments, and concluded that it possessed a special affinity for trypsin. Some writers have asserted that the serum is more active against trypsin of the same species, but Weil (8) and others have disproved this.

Morgenroth (9) thought that he obtained a specific antirenin by inoculating animals with renin, while Achalme (10) states that he produced a specific anti-trypsin by inoculating animals with trypsin. Other investigators repeated the work of Morgenroth and Achalme and have failed to confirm their results. Hamburger (11) showed that sodium chloride destroys pepsin in a neutral solution, thereby invalidating the evidence that a specific anti-pepsin is present in the blood; immune bodies are active only in a neutral or slightly alkaline reac-

\* Received for publication, March 7, 1914.

tion. We must conclude, then, that sufficient proof has not been advanced to support the view that the ferment-inhibiting substances of the blood are true antibodies.

Hedin (12) has shown that charcoal will bind trypsin and that the binding is proportional to the quantity of charcoal and to the time of interaction. Strong acids and alkalies quickly destroy trypsin, but Chittenden and Cummins (13) found it to be much more resistant to these agents in the presence of protein. Kudo (14) states that all the mineral acids are active in this respect, sulphuric acid being active in a dilution of one in a thousand, while the organic acids are much less so. Bayliss (15) and Abderhalden and Gigon (16) found that some of the products of tryptic activity possess the property of inhibiting the action of the ferment, the free amino acids being more active than the polypeptids.

Schwartz (17) concluded that the antitryptic activity of the serum is due to the lipoids. He found that he could partially inactivate sera by extracting them with ether, and that the sera could be reactivated by the addition of lecithin. Sugimoto (18) observed a decrease in the antitryptic strength of sera after extraction with ether and benzol, and concluded that the lipoids were the active constituents. He believes that a complete extraction of the fat, and the dissociation of the lipoid-protein combinations will cause a removal of the ferment-inhibiting action. He also tested alcoholic extracts which were prepared from egg-white, beef brain, and beef liver, and found them inactive as anti-enzymes when used as emulsions in solutions of sodium chloride. Meyer (3) suggests that the effect of the ether was due to the destruction of the anti-enzymes and not to their extraction. He also refers to the normal antitryptic index found in the lipoid-rich sera of luetics and diabetics. He found that it was not destroyed by extracting the dried serum with ether, and the fluid serum with petroleum ether; but states that this does not disprove the possibility of its being a lipoid-protein combination. He does not believe that protein cleavage products are the inhibiting agents. Cobliner (19) assumes that lipoids are not the active agents, as dried sera extracted with ether, chloroform, or petroleum ether do not lose their antitryptic activity. He found that the inhibiting action was increased if the serum was shaken with olive oil. Kirchheim (20) concludes that the antifermenit merely prolongs the action of the trypsin and does not destroy it. He observed that chloroform reduced the inhibiting action of the serum.

Rosenthal (21) believes that the action of the serum is due to protein cleavage products, and that its increase in carcinoma and other diseases with cachexia can be thus explained.

Two years ago we observed that sera preserved with chloroform soon lost their antitryptic action. The same observation had been made previously by Delezenne and Pozerski (22), but we did not know of it. This discovery convinced us that the ferment-inhibiting substances of the serum were lipoids, and that they were soluble in fat solvents. We (23) have published the results of some preliminary work based on this idea, in which it was shown that the soaps of the unsaturated fatty acids obtained from various sources possessed the property of acting as anti-enzymes. These results caused us to study the ferment-inhibiting action of the blood to see if it was due to the same agents.

The Fuld-Gross technique was used by most of the investigators engaged in the study of the anti-enzymes of the blood. We have already discussed in

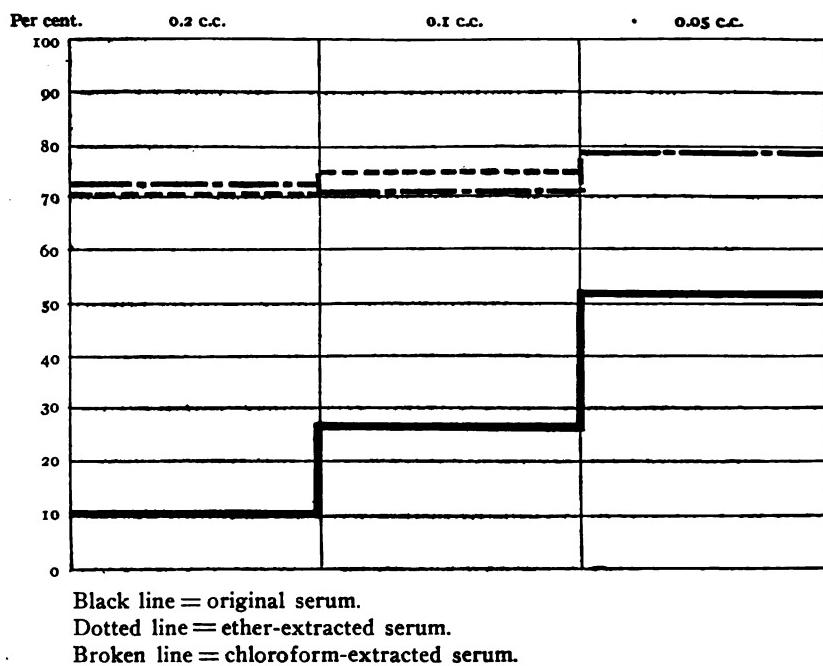
another paper (23) the difficulties we encountered in the use of this method. In this work, as well as in that previously reported, our interpretations were based upon the total incoagulable nitrogen determined according to the method recommended by Folin.

INFLUENCE OF CHLOROFORM OR ETHER EXTRACTION  
ON SERUM ANTITRYPsin.

Considerable work has been done with lipoid solvents in order to show the relation which exists between the lipoids of the serum and antitrypsin, but with contradictory results. In most of the work the solvent was allowed to act for a short time only, and, in some instances, on the dried serum. In the first experiment we wished to determine the influence of chloroform or ether on the enzyme-inhibiting action of the serum. Fresh dog serum was diluted 1 to 5 with salt solution, and divided into three parts. To one part was added chloroform; to another ether; the remaining portion was preserved without the addition of any agent. The three flasks containing the sera were then kept at room temperature for forty-eight hours. The mixtures were shaken frequently during this time. Before testing, the excess of chloroform or ether was removed with pipettes, and the serum then filtered through filter paper until clear. They were mixed in various dilutions with trypsin and placed in the incubator for thirty minutes. Casein was then added and the mixture incubated for two hours at 37° C. A control flask containing only trypsin and casein was incubated for the same period of time. The lines on text-figure 1 show the amount of digestion in each flask as compared with that obtained in the control tube which contained no serum. This method will be followed in all the subsequent experiments.

Text-figure 1 shows the results of extracting the serum with ether or chloroform. The lines in the text-figure show that in the tube containing 0.2 of a cubic centimeter of the unextracted serum, the digestion was only 10 per cent. of that obtained in the control, while in the tubes containing the extracted serum, the digestion was 70 per cent. of the control. In other words, the chloroform and ether had removed 60 per cent. of the inhibiting action of the serum. Dog serum has not the high anti-enzyme power of human serum, and this accounts for the comparatively large amount

used. This experiment has been repeated many times with human serum, horse serum, dog serum, and guinea pig serum, and similar results have been obtained with each. It was found necessary to shake the mixtures of chloroform and ether frequently, otherwise the inhibiting action of the serum was not removed. At least twice as much solvent as serum should be used.



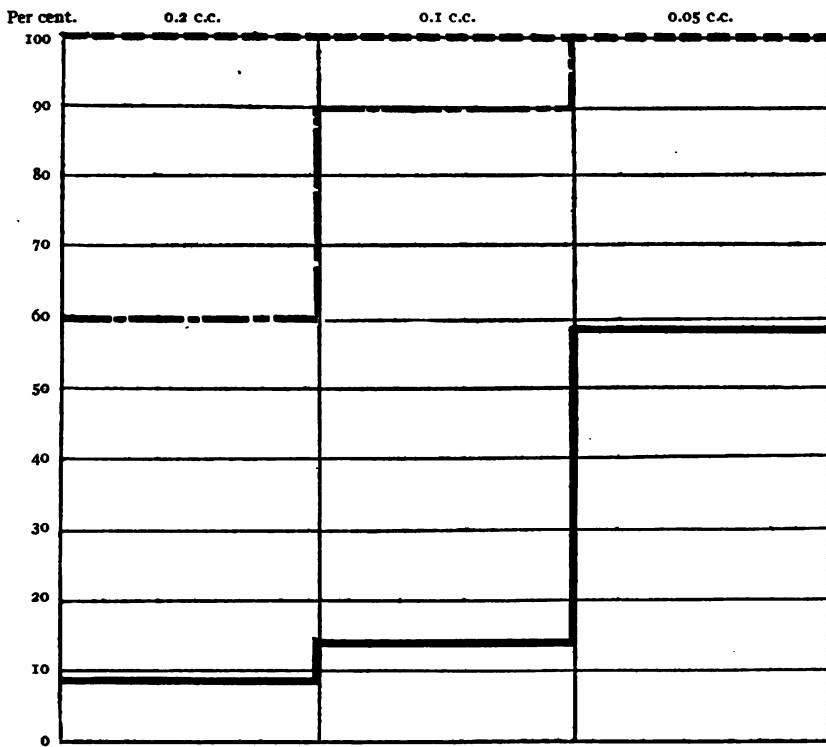
TEXT-FIG. 1. Effect of ether or chloroform extraction on serum antitrypsin.

Two possibilities must be considered in explaining the action of chloroform or ether on the enzyme-inhibiting action of the serum: first, that the substances causing the inhibiting action have been destroyed; and, second, that they have been taken up by the solvents.

#### INFLUENCE OF THE REACTION ON EXTRACTED SERA.

The next experiment was conducted to see if the activity of the serum could be restored by rendering it neutral or slightly alkaline in reaction. In order to duplicate the conditions present in the last experiment, ether was added to the serum in two tubes, and the

mixtures were kept at room temperature. A third tube of serum was kept without any preservative. After four days the ether was evaporated from one tube, the serum shaken thoroughly, and then made slightly alkaline with a dilute solution of sodium hydrate. This was done to see if the acids could be saponified, and the activity



Black line = human serum.

Dotted line = ether-extracted human serum.

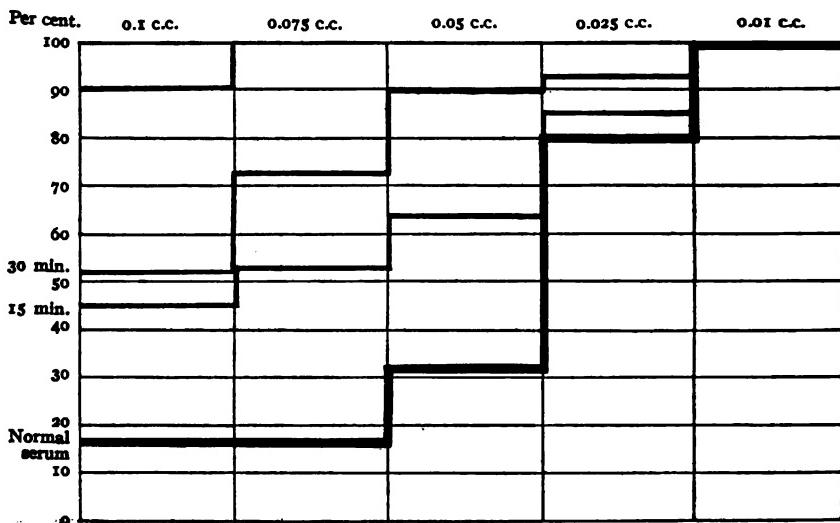
Broken line = ether-extracted human serum, ether evaporated.

TEXT-FIG. 2. Effect of evaporating ether extract and partial reactivation of serum.

of the serum restored. The ether was removed from the second tube by means of a pipette, and the serum was then filtered several times through filter paper. It was made neutral by adding a dilute solution of sodium hydrate. Text-figure 2 shows the results obtained in this experiment. The chart shows that only 8 per cent.

of digestion was obtained in the flask containing 0.2 of a cubic centimeter of the untreated serum, while 100 per cent. of digestion was obtained in the flask containing the same amount of ether-extracted serum. The serum which had been made slightly alkaline after evaporation of the ether showed about half the inhibiting action possessed by the one to which nothing had been added. These results indicate that the solvent does not destroy the anti-enzyme.

We must assume that the action of the alkali is due to a combination with some substance soluble in ether, as the alkali did not re-



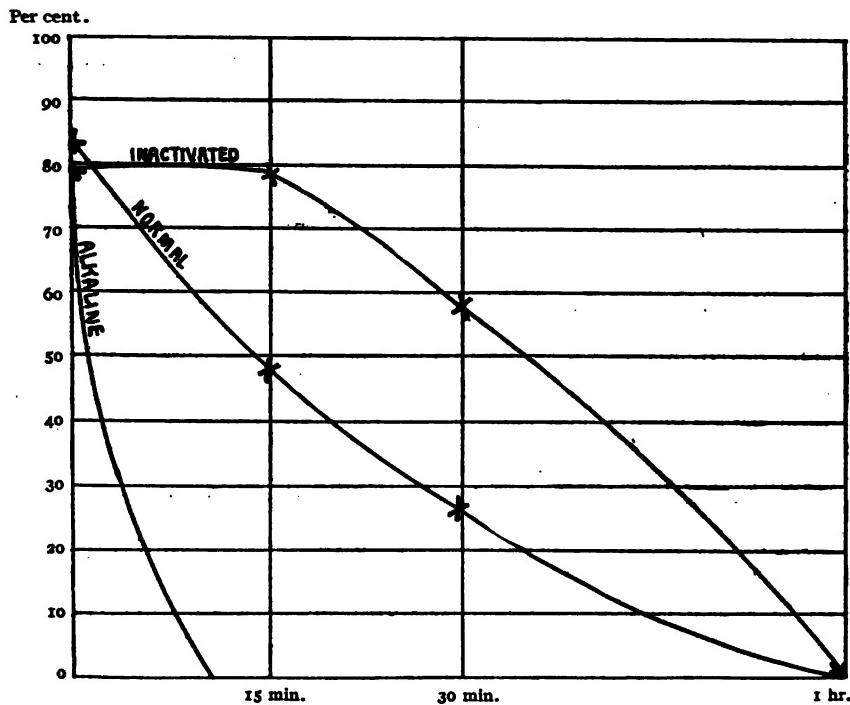
TEXT-FIG. 3. Effect of chloroform on serum antitrypsin at 37° C.

store the activity of the serum from which the ether had been removed, but partially restored it to the serum from which the ether was evaporated. Under these conditions the alkali would not be likely to combine with lipoids such as lecithin, cholesterol, etc., and so only the fatty acids remain. It cannot be expected that the full strength of the inhibiting action observed in the untreated serum should be obtained here. Following the evaporation of the ether, the extractives would be more or less insoluble in the serum and this would render it impossible to saponify all the free fatty acids without the aid of heat and an excess of alkali. If the removal of the inhibiting action at room temperature by chloroform is aided by the action of the serum lipase, a shorter time should be required

by incubating the serum at  $37^{\circ}$  C., ferments being more active at this temperature.

#### TIME REQUIRED FOR CHLOROFORM EXTRACTION AT $37^{\circ}$ C.

In the next experiment we wished to determine the length of time required for chloroform to remove the ferment-inhibiting action of the serum when the mixture was kept at  $37^{\circ}$  C. Chloroform was added to fresh serum, thoroughly shaken, and the



TEXT-FIG. 4. Rate of removal of serum antitrypsin in normal, inactivated, and alkalinized serum at  $37^{\circ}$  C.

mixture was placed in the incubator at  $37^{\circ}$  C. At fifteen minute intervals some of the serum was removed and tested for its ferment-inhibiting properties. In separating the serum from the chloroform it is necessary to centrifuge the emulsion at high speed for about five or ten minutes, and then to filter it through coarse filter paper several times until the serum is quite clear and all chloroform evapo-

rated. This experiment demonstrates that the ferment-inhibiting action of the serum is almost wholly lost after sixty minutes' incubation with chloroform (text-figure 3).

In the next experiment we wished to determine if lipase was essential to the removal of the inhibiting substances by the chloroform. The serum was divided into three portions. One portion was heated at 54° C. for thirty minutes in order to destroy the lipase; the second portion was made distinctly alkaline in order to prevent the serum from becoming acid; and the third portion was used as a control. Chloroform was added to all three portions and they were then placed in the incubator. A portion of each lot was removed at fifteen minute intervals and tested against trypsin for its anti-enzyme properties (text-figure 4).

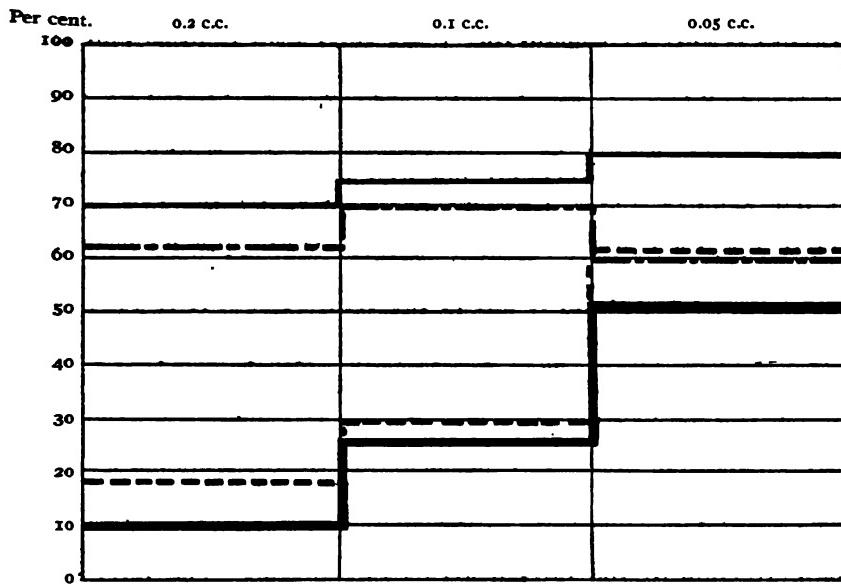
The results show that lipase is not essential to the removal of the inhibiting substances by the chloroform, and indicate that the inhibiting substances are probably fatty acid esters which are soluble in chloroform and ether. There was very little decrease in the inhibiting action of the inactivated serum during the first fifteen minutes of incubation. This may have been due to the absence of the lipase, or to some physical change in the serum caused by heat.

#### FATTY ACIDS AS ANTIFERMENTS.

The results obtained in the previous experiments indicate that the inhibiting agents are compounds of the fatty acids, and the next experiment was planned to see if this was the case.

Ether was added to fresh dog serum and the mixture was allowed to stand for two days at room temperature. The ether was then removed and divided into two portions. One portion was made slightly alkaline with sodium alcoholate and evaporated to dryness. The soaps were then dissolved in water and filtered; the acids liberated with hydrochloric acid, taken up in ether, and resaponified. The second portion of ether was shaken with water and the ether then evaporated at low temperatures. The soaps prepared from the first portion, the emulsion prepared from the second, and the serum from which the ether extracts were obtained, were tested for their ferment-inhibiting action. The original untreated serum was used

as a control. Text-figure 5 shows the percentage of casein digested by trypsin after it had been incubated for thirty minutes with these preparations. The lines on the chart show that the soaps prepared from the ether extract cause nearly as much inhibition as the untreated serum, while the water emulsion gives 62 per cent. of diges-



Heavy black line = original serum.

Lighter black line = extracted serum.

Dotted line = ether extract saponified.

Broken line = ether extract emulsified.

TEXT-FIG. 5. The antitryptic effect of the ether extract of normal serum when saponified.

tion. The inhibition caused by the emulsion is probably due to some of the acids combining with the alkali which had been used in the preparation of the casein solution. The soaps thus formed would of course inhibit ferment action. The original untreated serum gave 10 per cent. of digestion, and that extracted with ether 70 per cent. Complete removal of the inhibiting action of the serum is rarely obtained in less than four days' extraction at room temperature, and then, as a rule, only with chloroform.

Up to the present time all observers have failed to secure any

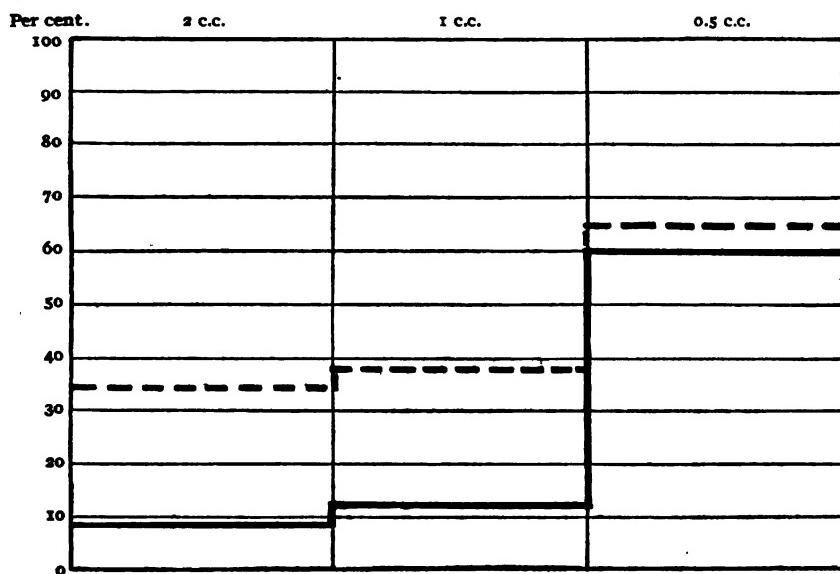
antitryptic effect of the ether or chloroform extracts. This is due solely to the fact that in the simple emulsions with which they worked, the intimate contact of the trypsin and the unsaturated fatty acid complex, which is the basis of the antitryptic activity, is not brought about. In the native serum these lipoids are in a state of dispersion sufficiently great to permit such contact, and we can reproduce it only by saponifying these fatty acid radicals and thus bringing them into a colloidal solution again. We have found that we can at all times secure the antitryptic effect in almost its original value from the extracts, if care is taken to secure complete saponification. If such extracts are not well saponified, so that droplets of unsaponified material can be observed under the high power microscope, then either little or none of the antitryptic effect will be recovered. The results of this experiment indicate that the inhibiting action of the serum is due to the fatty acids. It is not necessary to assume that they can act only as soaps; probably the esters containing unsaturated fatty acids are just as effective.

**REMOVAL OF ANTIFERMENT BY FILTERING ACID SERUM THROUGH  
KAOLIN.**

The results that we have been discussing were probably due to the extraction of the fatty acids by the solvent. The fatty acids can also be liberated by means of other acids, in which case they should be thrown out of solution and removed by filtration. The next experiment was planned to find out if the inhibiting substances could be removed by filtering through kaolin serum which had been made strongly acid with hydrochloric acid.

Twenty cubic centimeters of fresh dog serum were diluted with forty cubic centimeters of water, and five cubic centimeters of N/10 hydrochloric acid were added. The mixture was shaken thoroughly, allowed to stand for a few minutes, and then filtered several times through kaolin. After standing for a short time to permit all the serum to drain off, the kaolin was heated with sodium alcoholate. The mixture was then filtered and the filtrate evaporated to dryness. The soaps were dissolved in water and tested for their ferment-inhibiting properties. The acid serum obtained after filtration was made neutral and also tested. Some of the original unacidified

serum was filtered through kaolin to see if kaolin filtration would exert any influence on normal serum. Text-figure 6 shows the results of filtering acidified serum through kaolin. The inhibiting action of the serum which was made acid and then filtered through kaolin was completely lost, and is therefore not shown in the chart. The soaps prepared from the kaolin were active as inhibiting agents



Black line = serum diluted by 10 (horse).

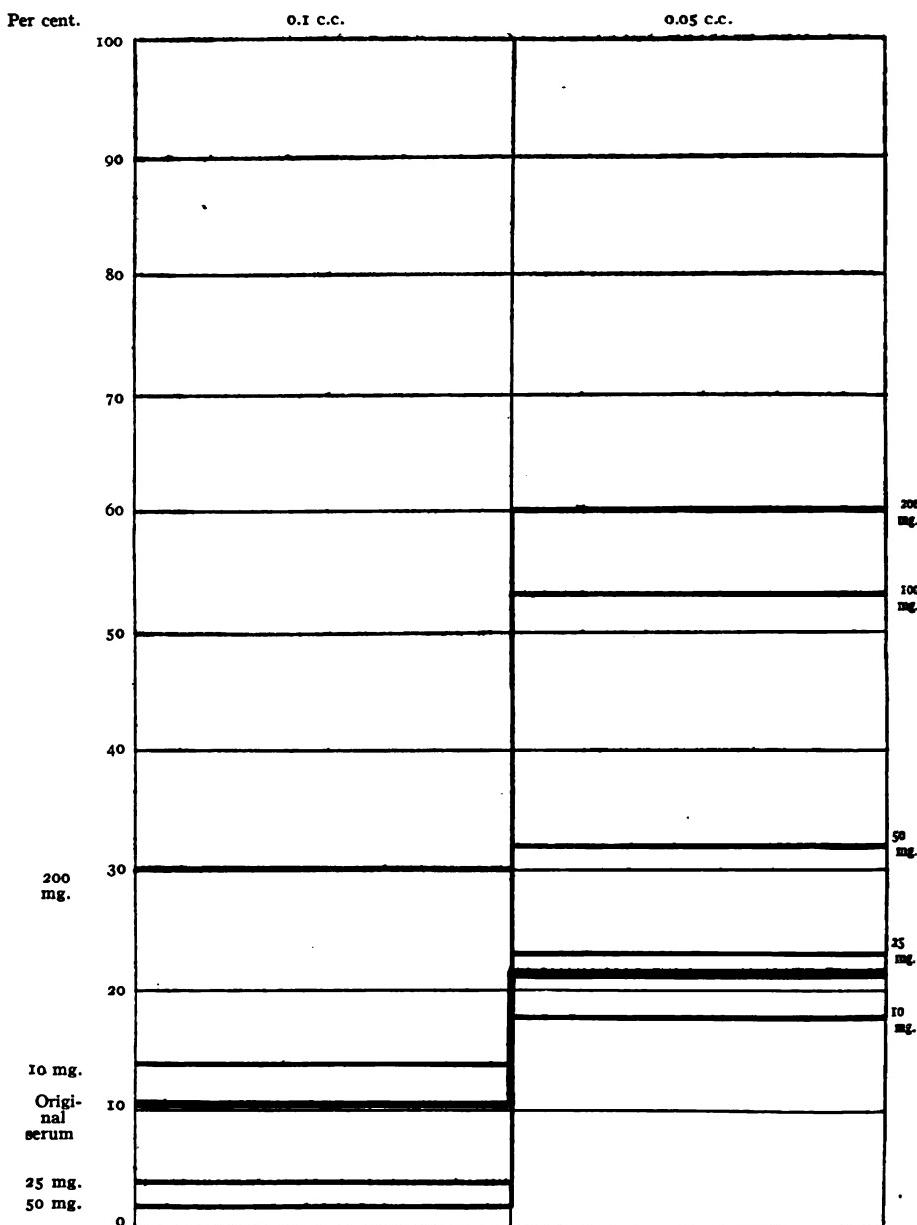
Dotted line = kaolin extract saponified, *i.e.*,  $\frac{1}{10}$  strength of serum.

TEXT-FIG. 6. Antitryptic effect of kaolin extract saponified.

and indicate that the fatty acids are the active substances present in the serum. The strength of the soap solution was about 0.1 of that of the untreated serum. We have not been able by this method to recover a larger amount of the inhibiting substances, though the serum has usually lost all its anti-enzyme properties.

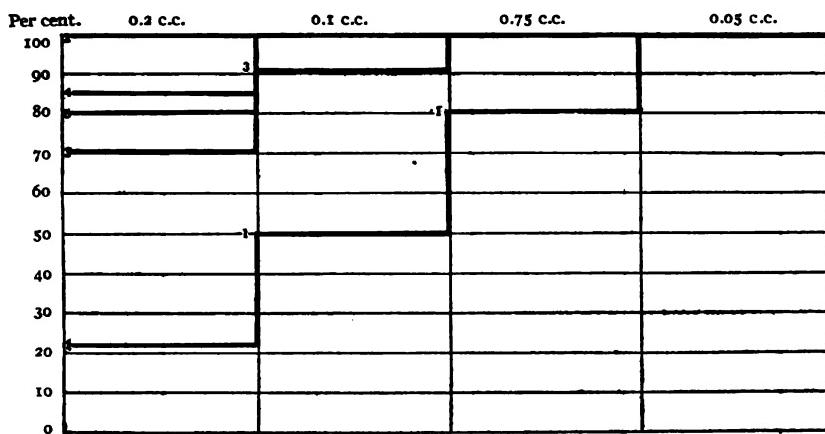
#### INFLUENCE OF IODIN OR POTASSIUM IODIDE ON ANTIFERMENT.

In our previous work (23) we have shown that soaps of the unsaturated fatty acids lose their ferment-inhibiting properties when treated with iodin. For this purpose we used iodin dissolved in a solution of potassium iodide and removed the excess of iodin by



TEXT-FIG. 7. Effect of varying amounts of potassium iodide on serum antitrypsin.

shaking the mixture with chloroform. This last step was necessary, as iodin inhibits tryptic activity. In the present work we were unable to use the iodin-iodide of potassium mixture, as we have shown that serum loses some of its ferment-inhibiting properties even when shaken with chloroform for a short time. For these reasons we have first investigated the action of potassium iodide alone on the ferment-inhibiting action of the serum.



1 = dog serum, 3 days at room temperature.

2 = 1 c.c. dog serum + 200 mg. potassium iodide, 3 days at room temperature.

3 = 1 c.c. dog serum + 100 mg. potassium iodide, 3 days at room temperature.

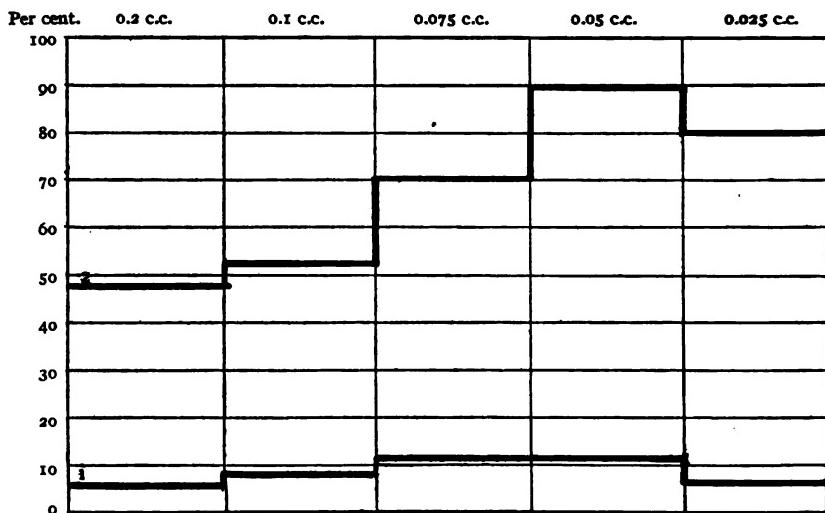
4 = 1 c.c. dog serum + 200 mg. sodium iodide, 3 days at room temperature.

5 = 1 c.c. dog serum + 100 mg. sodium iodide, 3 days at room temperature.

TEXT-FIG. 8. Effect of sodium iodide and potassium iodide on antitrypsin *in vitro*.

Fresh human serum was divided into six portions of five cubic centimeters each. To one of these portions, potassium iodide was added in the proportion of 10 milligrams to one cubic centimeter; to the second, 25 milligrams to one cubic centimeter; and to the third, 50 milligrams; to the fourth, 100 milligrams; and to the fifth, 200 milligrams. The sixth received no potassium iodide and was used as a control. The tubes containing the sera were placed in the incubator for three hours and afterwards kept at room temperature over night. The following morning they were incubated for thirty minutes with trypsin, and the casein was then added. Control tubes containing trypsin and potassium iodide, without serum, showed

that the chemical did not inhibit the activity of the trypsin. Text-figure 7 shows the influence of potassium iodide on the ferment-inhibiting action of the blood. The chart shows that potassium iodide even under these conditions destroys some of the ferment-inhibiting action of the serum. In the first column in which 0.1 of a cubic centimeter was used, the influence was not marked; but in the second column in which 0.05 of a cubic centimeter of serum was used, there was considerable diminution of the inhibiting action.



1 = guinea pig serum (1 c.c. diluted by 10), normal.

2 = guinea pig serum (1 c.c. + 200 mg. potassium iodide), over night in incubator.

TEXT-FIG. 9. Effect of potassium iodide on antitrypsin of guinea pig serum.

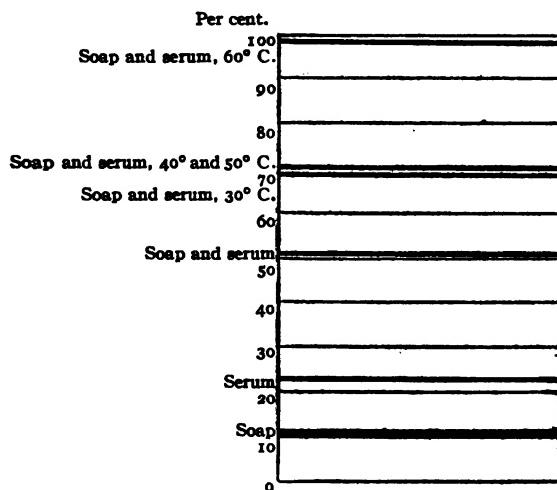
Another experiment was made to see if other iodides would act in a similar manner. In this instance fresh dog serum was treated with sodium iodide and potassium iodide. The technique used was the same as that described for the preceding experiment except that the mixtures were kept for two days at room temperature. The results of this experiment (text-figure 8) are more striking than those of the preceding one. This may be due to the fact that the iodides were in contact with the serum twenty-four hours longer. The sodium iodide is just as active as the potassium iodide in removing the inhibiting action of the serum.

Still more conclusive are the results obtained with guinea pig serum, as this serum is more active as an inhibiting agent. The mixture of serum and potassium iodide stood in the incubator over night. Text-figure 9 shows the influence of potassium iodide on guinea pig serum.

The effect of potassium iodide on the serum antitrypsin seems to be most marked on fresh sera. Treatment of serum with an equal volume of strong solution of hydrogen peroxide, and incubated over night, also causes complete loss of its antitryptic power.

#### INFLUENCE OF HEAT.

Serum loses its ferment-inhibiting properties if it is heated for thirty minutes at 65° C. If the fatty acids are the inhibiting agents, they must undergo some physical or chemical change which renders them inactive. In the next experiment we wished to determine the



TEXT-FIG. 10. Effect of heat on the antitryptic activity of soap and serum.  
1 c.c. contains 0.1 c.c. of serum and 0.01 gm. of soap.

influence of heat on the ferment-inhibiting properties of soap when it is mixed with serum. We had found that soaps dissolved in water did not lose their activity when heated at 100° C., and so it was of interest to determine if they were more susceptible in the presence of serum protein.

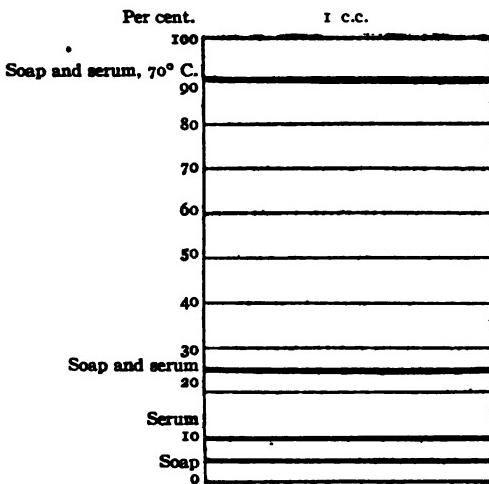
Linseed oil soap, with an iodin value of 110, was used in this experiment. A 10 per cent. solution of dog serum was prepared and to this was added an equal amount of a 1 per cent. soap solution. Portions of the mixture were heated for thirty minutes at 30°, 40°, 50°, and 60° C., and then tested for their ferment-inhibiting properties. The unheated mixture of soap and serum and the unheated soap and serum solutions were tested separately as controls (text-figure 10). The chart shows that the soap solution was active as it permitted only 12 per cent. of digestion, while the serum was less active, permitting 23 per cent. of digestion. The unheated mixture of soap and serum gave 52 per cent. of digestion, while the mixtures heated at 30°, 40°, and 50° C. gave 70 per cent. In the mixture heated at 60° C. the inhibition of ferment action was completely lost.

The amount of inhibition obtained with the unheated mixture of soap and serum was less than that obtained with the soap or the serum alone. This indicated that some of the inhibiting action was destroyed by mixing the serum and soap. In our previous work we found that a slight degree of acidity was sufficient to destroy the activity of the soaps and so we immediately tested the serum used in this experiment. It was found that one cubic centimeter of the serum required 0.1 of a cubic centimeter of an N/10 sodium hydrate solution to neutralize it. This explains why the mixture of soap and serum was less active than the soap or serum separately. The high degree of acidity was probably due to the fact that the serum was several weeks old.

Experiments were then conducted to determine the influence of heat on soap mixed with neutral serum. The same acid serum was made slightly alkaline to phenolphthalein by means of a sodium hydrate solution. Text-figure 11 shows the influence of heat on linseed oil soap when mixed with neutral serum.

Here again the unheated mixture of soap and serum does not inhibit the action of trypsin as much as the soap or the serum alone, though it does show more inhibition than when the serum was acid in reaction. It is possible that here, also, there was some dissociation of the soaps which caused a decrease of inhibiting action. Heating at 70° C. caused almost complete loss of activity of the soap-serum mixture.

Further experiments were made with serum which had been rendered distinctly alkaline by adding 0.1 of a cubic centimeter of N/10 sodium hydrate solution to the neutral serum. This alkaline serum-soap mixture lost only 30 per cent. of its inhibiting proper-



TEXT-FIG. II. Effect of heat on the antitryptic activity of soap and serum in neutral reaction.

ties when heated for thirty minutes at 70° C. In other words, loss of activity as the result of heating appears to bear some relation to the reaction of the serum.

#### DISCUSSION.

The results obtained in the preceding experiments justify, we think, the conclusion that the ferment-inhibiting properties of the serum are due to the presence of compounds of the unsaturated fatty acids. Their solubility in ether or chloroform suggests that they are not in the form of soaps, but are present as esters. In some experiments which are not described in this paper, we found that a fine emulsion of olein in serum inactivated by heat, or in salt solution, possessed some ferment-inhibiting action, the inhibiting action bearing a distinct relation to the degree of dispersion. This observation is significant as numerous investigators have reported that the antitrypsin of the serum is increased after eating.

In our study of the ferment-inhibiting substances present in tuberculous caseous material (24) we at first thought that the unsaturated fatty acids were present in the form of soaps, but subsequent experiments showed that they could also be extracted by means of chloroform or ether, after which the material was digestible by trypsin. These results indicate that here also the acids in the form of esters are active as inhibiting agents.

If fresh serum is tested daily for its ferment-inhibiting action it will be found that its strength decreases from 5 to 10 per cent. for the first few days; after that it remains almost constant for an indefinite period of time. With this decrease in anti-enzyme activity there is an increase in acidity. The acidity is probably due to the liberation of fatty acids by the action of the serum lipase on the fatty acid compounds present. If serum lipase is mixed with ethylbutyrate and then incubated, the degree of acidity will be found to be practically the same at the end of four hours as after twenty-four hours. But if the mixture is made neutral at the end of the first four hours and again incubated, the acidity at the end of the second four hours will be almost equal to that obtained after the first incubation. In other words, the ferment can act to a certain point only; after that its action is inhibited either by the amount of acids liberated or by the synthetic compounds formed from the acids. When chloroform or ether is added to the serum, the acids are taken up by the solvent almost as fast as they are liberated, and so they do not interfere with the action of the lipase. Under these conditions the ferment remains active until most of the substrate consisting of the fatty acid compounds has been destroyed. We have found that chloroform acts better than ether in removing the inhibiting agent, and this is to be expected as chloroform is known to exert less influence on ferment activity. This hypothesis also explains why a serum can be left for months with other preservatives without losing all its antiferment properties. In order to remove the inhibiting substances with chloroform it is necessary that a large amount of the solvent be used, and that the mixture be shaken frequently.

Two possibilities must be considered in explaining the destruction of the ferment-inhibiting properties of the serum by heat: first,

it might be due to saturation of the unsaturated fatty acids; second, to the mechanical inclusion of the fatty acid compounds in the fine coagula caused by the heating. If the fatty acids become saturated when heated with protein the combination formed must be easily dissociated, as their activity is not permanently lost. If an inactive soap-serum mixture is saponified with strong solutions of sodium hydrate, and the fatty acids are then liberated in the usual manner and resaponified, it will be found that the soaps are just as active as those obtained from an unheated portion of the same mixture. At first we thought the destruction of the inhibiting action was due to the mechanical inclusion of the fatty acid compounds in the fine coagula caused by heating, but subsequent experiments with sera made strongly alkaline with sodium hydrate disproved this. Under these conditions, coagula would not form, yet the resistance of the inhibiting substances to heat was greatly increased. We have noticed frequently that there is an increase of from 5 to 20 per cent. in the antiferment activity of sera heated for fifteen minutes at 54° to 55° C., especially in sera that are lipemic.

Thiele (25) found that the neutral fat of chyle was adsorbed by serum when the mixture was kept for several hours at 37° C. The fat in such a mixture could not be extracted with ether, but the combination could be broken up by peptic digestion and by treatment with alcohol. A similar explanation may apply to our observation that a soap-serum mixture heated at 70° C. loses its anti-enzyme properties. The iodin values of the heated and unheated mixtures are the same. We must therefore assume that the combination formed is one which is readily dissociated.

The effect of the potassium iodide is probably due to the liberation of small amounts of free iodin which can then saturate the unsaturated carbon bonds. Thus the iodin value (determined by precipitating the serum proteins by alcohol and titrating the alcoholic filtrate) of a serum treated with potassium iodide falls to almost one fifth of the original value. Free iodin dissolved with a small amount of potassium iodide also lowers the antitryptic activity. The relation of the iodide to serum antitrypsin will be discussed in a later paper. The influence of iodin in removing the inhibiting action of the serum shows that the inhibiting agents belong to the unsaturated group of the fatty acids.

The effect of hydrogen peroxide in completely destroying the antitryptic activity; the fact that the extracted lipoids, which are the active agents, are all unsaturated compounds, having an iodin value of from fifty to sixty; and also the fact that sera with a high antitryptic index give a higher iodin value than sera with a low antitryptic index, all tend to confirm this view from a chemical standpoint. From the clinical investigations so far reported there is similar confirmation; there is an increase of the anti-fermentation after eating; in the acute infections such as typhoid fever, pneumonia, etc.; in chronic infections such as syphilis and tuberculosis; in Graves' disease; and in the severe anemias, such as those accompanying malignant growths, etc. In all but the first of these conditions, the demands of the body or the action of the toxins, cause a marked inroad into the fat depots of the body. This disappearance of fat in conjunction with a lowering of the oxidation powers of the liver and tissues in general causes an increase in the amount of unsaturated fatty acids in the blood and thus an increase in the ferment-inhibiting action.

#### CONCLUSIONS.

1. The ferment-inhibiting action of the serum is due to the presence of compounds of the unsaturated fatty acids.
2. These fatty acid compounds may be removed from the serum by means of chloroform or ether.
3. Soaps prepared by saponifying the chloroform or ether extracts inhibit the action of trypsin.
4. The anti-enzyme action of the serum can be removed by filtering acid serum through kaolin, and can in part be restored by extracting the kaolin.
5. The decrease in strength of anti-enzyme in old sera is probably due to the action of the serum lipase.
6. Iodin, potassium iodide, or hydrogen peroxide remove the inhibiting action of the serum.
7. Soaps of the unsaturated fatty acids lose their ferment-inhibiting action when heated with serum at 70° C.

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JANUARY

## SEROTOXIN.

### STUDIES ON FERMENT ACTION. XIV.\*

BY JAMES W. JOBLING, M.D., AND WILLIAM PETERSEN, M.D.

*(From the Department of Pathology of the College of Physicians and Surgeons,  
Columbia University, New York.)*

In a previous paper (1) we have demonstrated the lipoidal nature of serum antitrypsin and have discussed the various methods which may be employed to remove the antitrypsin from the serum; *i. e.*, by extraction, or by the saturation or oxidation of the unsaturated carbon bonds of the fatty acids on which the antityptic property depends. In view of the fact that practically all sera contain proteolytic ferments, we undertook to determine whether or not sera from which the protective substance had been removed, with resulting exposure of the serum proteins, would be toxic for the homologous animal; and if so whether complete removal of the lipoids was necessary to produce toxic effects. Apart from the interest that such a study would have in relation to the production of the so called anaphylatoxins, we felt that numerous pathological conditions, which at present are not wholly understood, might possibly have their basis in a protein intoxication,—a true auto-intoxication in the sense that the toxic substances were formed from the serum or cellular protein of the host, and without reference to the gastro-intestinal tract. We have in mind particularly such conditions as arteriosclerosis, nephritis, asthma, and acute acidosis in infants. The work of Longcope (2) supports this idea. He observed that repeated injections of egg albumen into sensitized dogs and cats caused nephritis and other organic lesions.

#### TOXICITY OF SERA WHICH HAVE BEEN RENDERED ACID AND THEN FILTERED.

Our first experiments were made with serum from which the lipoids had been removed by acidifying the serum and then filter-

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ing it through hard paper filters and kaolin, or through a Berkefeld filter. We have shown that part of the antitrypsin can be recovered from such filters by saponification.

0.2 of a cubic centimeter of N/10 sulphuric acid was added to each cubic centimeter of fresh guinea pig serum, and the mixture was filtered several times through a hard paper filter in the evening. It was then placed on ice over night, and on the following morning it was neutralized before injection. In all instances unless otherwise stated the injections were given intravenously.

Amount injected. Weight of guinea pig. Dose per gm. of weight.

4 c.c.	250 gm.	0.016 c.c.
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This dose given intravenously caused the immediate symptom complex of anaphylaxis with death of the animal in two minutes. The autopsy findings were typical,—lungs emphysematous, heart beating, and blood fluid.

In the next experiment 4 c.c. of N/4 hydrochloric acid were added to 20 c.c. of guinea pig serum and left over night in the ice box. Before injection it was passed through a Berkefeld filter.

Amount injected. Weight of guinea pig. Dose per gm. of weight.

5 c.c. (not neutralized).	330 gm.	0.015 c.c.
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There were marked respiratory convulsions, defecation, scratching of the nose, and the animal was weak for several hours, though it finally recovered.

Amount injected. Weight of guinea pig. Dose per gm. of weight.

5 c.c. (neutralized).	250 gm.	0.02 c.c.
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Typical death in three minutes. At autopsy the lungs were emphysematous, the heart was beating, and there were no intravascular clots.

In both of these experiments the serum from which the lipoids had been removed—not merely rendered inactive by acidifying—had become toxic for its own species in doses from 0.016 to 0.02 of a cubic centimeter per gram of weight. The simple acidification of the serum, which inactivates its antitryptic effect, does not seem to make the sera toxic in the same degree, probably because on re-injection the lipoids are again brought into a more active condition. Some toxic symptoms are, however, elicited by such sera in large doses (five cubic centimeters).

#### TOXICITY OF SERA AFTER EXTRACTION WITH ETHER OR CHLOROFORM.

We have shown that serum antitrypsin can be removed by ether extraction, and that the antitrypsin can be recovered in an almost quantitative relation from the ether extract by saponification of the extracted lipoids. That such sera are toxic is shown in the following experiments.

*Serotoxin.*

Guinea pig serum was placed under ether on Jan. 6 at room temperature and was left for two days. The flask was shaken occasionally.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
4 c.c.	330 gm.	0.012 c.c.

Death was immediate. The lungs were emphysematous, the heart had ceased beating, there was a firm clot in the right ventricle, and the gall bladder was distended.

A second preparation of serum was placed under ether on January 8 and permitted to stand at room temperature until January 13. The effect on intravenous injection was as follows:

Dose in c.c.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4 c.c.	330 gm.	0.012 c.c.	Immediate death. Lungs emphysematous. Heart filled with a large clot.
2 c.c.	245 gm.	0.008 c.c.	Immediate death. Findings as above.
1 c.c.	320 gm.	0.003 c.c.	Some respiratory spasm. Looked sick. Complete recovery.

In like manner chloroform will remove the serum antitrypsin, the result being even more rapid than with ether. It is best to mix about two volumes of pure chloroform with one volume of serum and to shake the mixture thoroughly, so that a thick emulsion is formed. After incubation or standing at room temperature the emulsion is centrifuged at high speed, and the supernatant serum is pipetted off and filtered through a coarse filter paper until the serum is quite clear and all the chloroform has evaporated. In the next experiment the serum was extracted with chloroform for two days at room temperature and the serum then removed.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
3.5 c.c.	185 gm.	0.018 c.c.

Death was almost instantaneous. The lungs were emphysematous, the heart beating, and the blood fluid.

A similar preparation was kept at room temperature for five days and then tested.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4.0 c.c.	280 gm.	0.014 c.c.	Immediate death.
2.0 c.c.	310 gm.	0.006 c.c.	Immediate death.
1.0 c.c.	320 gm.	0.003 c.c.	Immediate death.
0.5 c.c.	365 gm.	0.0013 c.c.	Respiratory spasm. Looked sick. Complete recovery.

## TOXICITY OF IODIZED SERA.

We have shown that the antitryptic effect of the serum can be completely destroyed by agents that oxidize or saturate the unsaturated bonds of the fatty acids, as for instance with potassium or sodium iodide, free iodin, or hydrogen peroxide. The iodide can not be used for the preparation of serotoxin because to lower the antitryptic index relatively large amounts must be used and these salts in themselves injected intravenously in small doses will produce toxic effects similar to the toxic effects of the serum.

Free iodin dissolved in serum containing only a trace of potassium iodide can be readily used for our purpose, according to the method described by von Dungern and Hirschfeld (3). With an excess of iodin a heavy precipitate is thrown out of the serum, but this is redissolved on the addition of an excess of sodium hydroxide, and the serum can then be brought back to a neutral reaction and will remain clear. It requires about two days at incubator temperature to remove completely the antitryptic action. Such sera remain sterile and will not autolyze if an excess of iodin is added. The results of an intravenous injection of an iodized serum which no longer had any antitryptic effect were as follows:

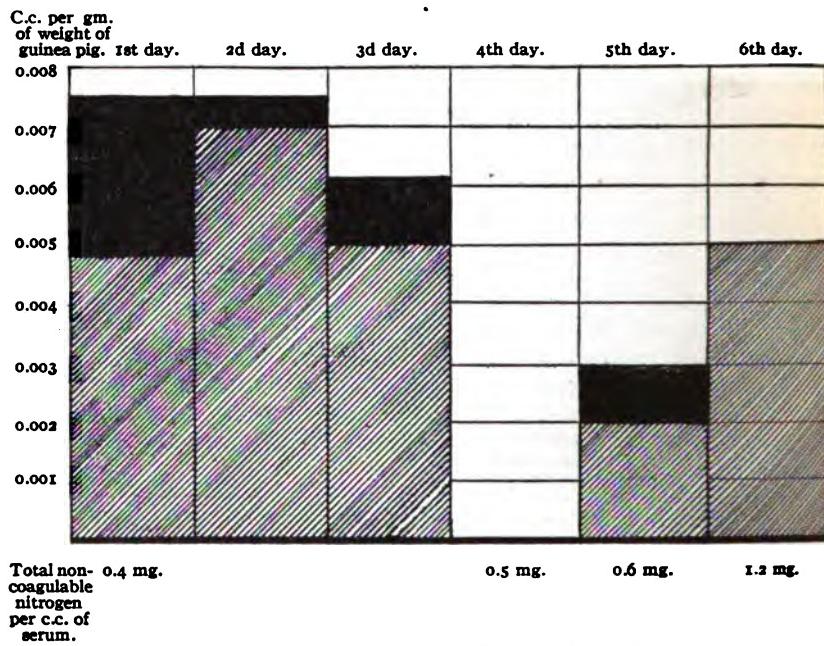
Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
3.2 c.c.	320 gm.	0.01 c.c.	Death in 2 min. Lungs emphysematous. Heart blood fluid. Heart beats strongly.
1.6 c.c.	310 gm.	0.005 c.c.	No effect.

Typical anaphylactic shock was produced with immediate fatal result in a guinea pig which had not been sensitized. This result is of course not comparable to the work of Bruck (4) and Friedberger and Ito (5), who have attempted to show that animals may be sensitized to iodized proteins. It is interesting to note that von Dungern and Hirschfeld (3) observed that their iodized sera underwent autolysis readily, but they could find no explanation for this fact, because they found no difference in the digestion of casein when iodized. They entirely overlooked the fact that the iodin might act on other bodies besides proteins. We have observed no increase in autolysis if an excess of iodin is present because the iodin

itself interferes with ferment action. The serum proteins are, however, digested more easily by trypsin after such treatment. We have been unable to secure toxic sera by treatment with strong hydrogen peroxide.

#### INFLUENCE OF TIME ON CHLOROFORM EXTRACTION.

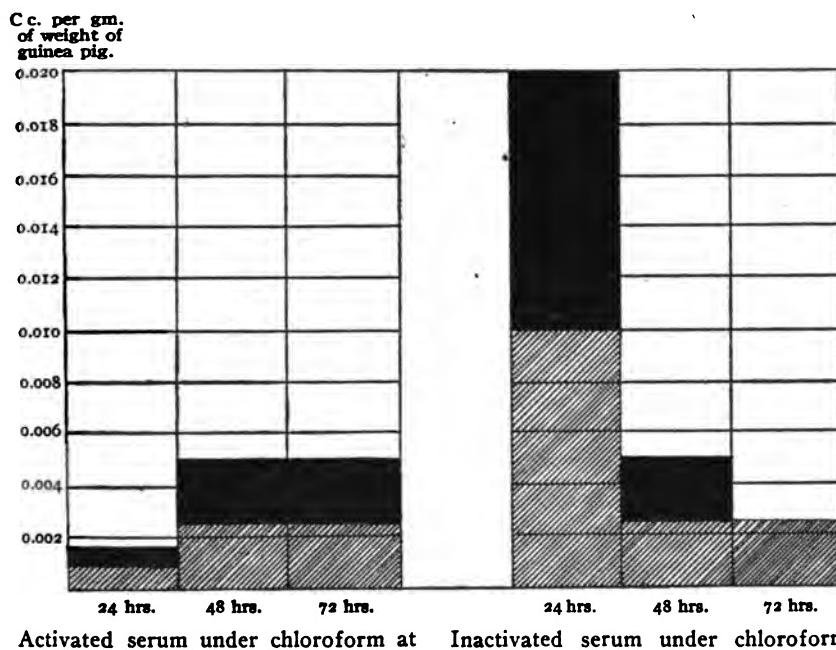
We next determined the length of time required for serum kept at room temperature under chloroform to become toxic. For brevity the result has been charted in text-figure 1. The serum before



TEXT-FIG. 1. Toxicity of guinea pig serum treated with chloroform at room temperature.

treatment was not toxic in large doses (5 c.c.), and the total non-coagulable nitrogen per cubic centimeter amounted to 0.2 of a milligram. As will be seen from the chart there was a progressive increase in toxicity after the first day until a maximum was reached on the fifth day, when the minimum lethal dose was 0.003 of a cubic centimeter per gram of weight, or 0.75 of a cubic centimeter for a guinea pig weighing 250 grams. There then follows a rather

sudden loss of toxicity, so that 0.005 of a cubic centimeter per gram of weight was no longer toxic. During this time there had been a constant autolysis of the serum, as indicated by the increase in the total non-coagulable nitrogen (Folin's method) from 0.2 of a milligram per cubic centimeter to 1.2 milligrams per cubic centimeter on the sixth day. In each case the minimum lethal dose caused the typical anaphylactic complex, although it was found that excessive



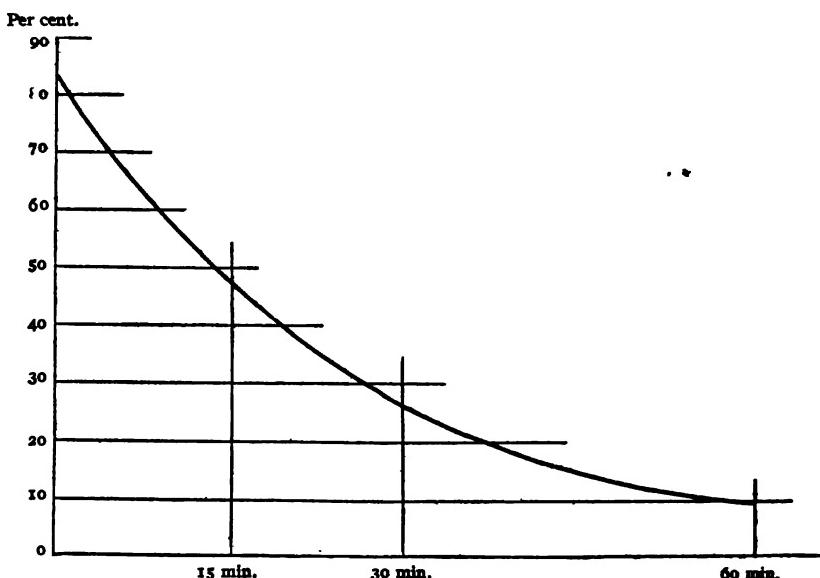
Activated serum under chloroform at 37° C.      Inactivated serum under chloroform at 37° C.  
Black = minimum lethal dose in c.c. per gm. of weight.  
Shaded = sublethal dose.

TEXT-FIG. 2. Toxicity of guinea pig serum treated with chloroform at 37° C.

doses, two minimum lethal doses, caused thrombosis in the larger vessels and heart.

When left at 37° C. the serum becomes toxic much earlier, and the atoxic stage is correspondingly produced at an earlier period of time (text-figure 2). The active serum so prepared has reached a very toxic stage in twenty-four hours, so that 0.0015 of a cubic centimeter per gram of weight of guinea pig is toxic,—0.31 of a cubic centimeter killed a guinea pig weighing 210 grams,—while the

inactivated serum (fifteen minutes at 54° C.) is toxic only in a much larger dose, 0.02 of a cubic centimeter per gram of weight. After forty-eight hours, however, the active serum has become less toxic, while the inactivated serum has become more toxic than before. Here, as in the previous experiment, there is a constant increase in the amount of non-coagulable nitrogen. In other experiments we have observed that the toxicity will reach its maximum within eight to twelve hours and will then rapidly decline. This depends entirely upon the amount and the rate of removal of the lipoid protective substances. Thus the inactivated serum becomes toxic less rapidly owing to the fact that the antitrypsin is removed more slowly than from the active serum at 37° C.



TEXT-FIG. 3. Curve showing the rate of extraction of serum antitrypsin by chloroform at 37° C.

The rate of removal of the antitrypsin from the serum by chloroform is shown in text-figure 3. In this experiment samples of serum were removed from the incubator at fifteen minute intervals, and the antitrypsin was determined by the method described in our previous papers (6).

0.075 c.c. of normal serum inhibited 84 per cent. of digestion.

After 15 minutes under chloroform it inhibited 48 per cent. of digestion.

After 30 minutes under chloroform it inhibited 27 per cent. of digestion.

After 1 hour under chloroform it inhibited 10 per cent. of digestion.

In one hour practically all the antitrypsin has been extracted. When made strongly alkaline this extraction is markedly accelerated, while the inactivated serum is more resistant to the chloroform extraction during the first half hour, although after about an hour's extraction the lipoids in this case are also removed. The serum is toxic, however, before all the antitrypsin is removed. This is shown by removing a sample after one half hour's incubation. These sera kill in a dose of about 0.01 of a cubic centimeter per gram of weight, and in this case the immediate cause of death is intravascular coagulation. There is no increase in the non-coagulable nitrogen at this time.

#### PROTOCOL I.

##### *One Hour Chloroform Serum.*

Dose.	Weight.	Dose per gm. of weight.	Result.
4.5 c.c.	300 gm.	0.015 c.c.	Immediate death.
3.2 c.c.	270 gm.	0.012 c.c.	Respiratory spasms. Prostrated for 30 min. Recovery.

##### *Two Hour Chloroform Serum.*

4.0 c.c.	260 gm.	0.015 c.c.	Immediate death.
1.6 c.c.	215 gm.	0.0075 c.c.	Death in 15 min. Lungs distended. Heart blood fluid. Heart beats.

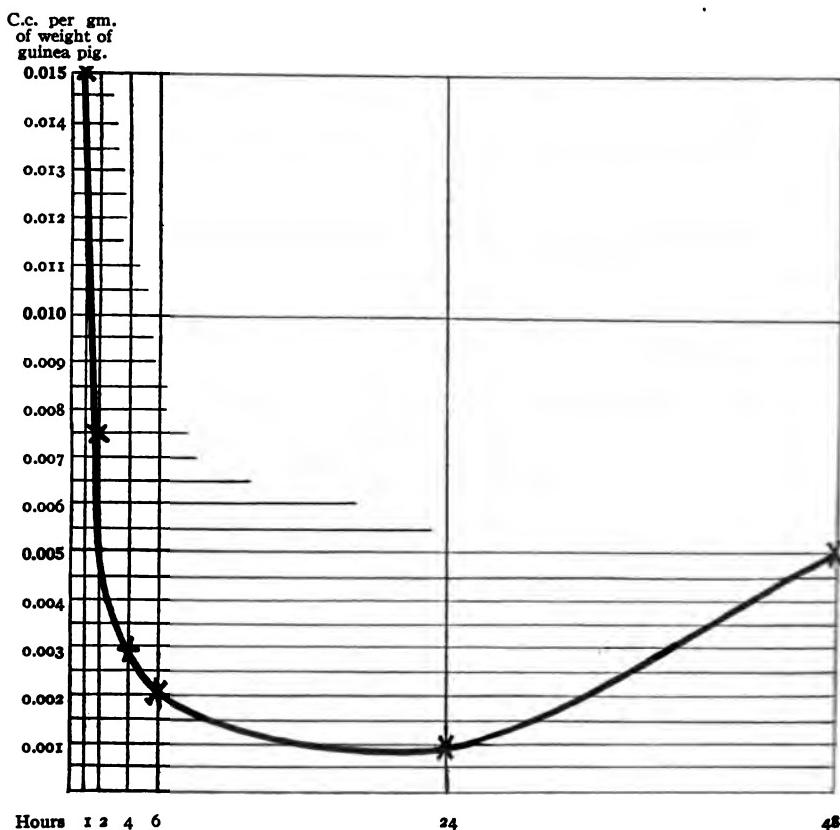
##### *Four Hour Chloroform Serum.*

1.7 c.c.	280 gm.	0.006 c.c.	Immediate death. Heart blood clotted. Heart does not beat. Lungs emphysematous.
1.0 c.c.	340 gm.	0.003 c.c.	Death in 30 min. Heart beats. Lungs somewhat distended.

##### *Six Hour Chloroform Serum.*

1.1 c.c.	370 gm.	0.003 c.c.	Immediate death. Heart blood clotted. Lungs distended.
0.62 c.c.	310 gm.	0.002 c.c.	Immediate respiratory spasms. Prostrated. Death in 20 min. Small clot in right heart. Blood fluid. Lungs slightly distended.

In text-figure 4 is shown the rate of increase of toxicity observed at 1, 2, 4, 6, 24, and 48 hour intervals of serum kept under chloroform. As will be observed there is a rapid increase during the first six hours, after which the toxicity remains at a constant



TEXT-FIG. 4. Curve showing the increase of toxicity of guinea pig serum treated with chloroform at 37° C. Minimum lethal dose in c.c. per gm. of weight.

level for a period of twelve or more hours, and then rapidly decreases. The minimum lethal doses have been charted from protocol I. The twenty-four and forty-eight hour tests were made at a different time, though with the same lot of serum.

## INTRAVASCULAR CLOTTING.

The minimum lethal dose of the toxic sera when given intraperitoneally is much larger, at least five to six times the intravenous dose being required. The immediate cause of death observed in the minimum lethal dose has been either a typical anaphylactic complex, with the lungs emphysematous, the heart beating, and the blood fluid,—observed in the acidified and filtered serum, in the iodized serum, and in the chloroform serum, especially when kept at room temperature; or death has been due to immediate intravascular coagulation with a secondary emphysema, observed in the ether sera, and in the chloroform serum prepared at 37° C. In order to rule out the effect of coagulation we have made the following experiments. The serum used in this experiment had been incubated for two hours at 37° C. The minimum lethal dose was then determined.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Remarks.
1.0 c.c.	340 gm.	0.003 c.c.	Weak. Prostrated. Recovered.
1.1 c.c.	220 gm.	0.005 c.c.	Immediate death. Intravascular coagulation.

Some of this serum was mixed with saline solution to which had been added 1 per cent. sodium citrate, and injected.

Dose.	Citrate.	Weight of guinea pig.	Dose per gm. of weight.
1.25 c.c.	1.25 c.c.	250 gm.	0.005 c.c.

Immediate death. Heart found beating. Lungs emphysematous. Small clot in right heart.

Inasmuch as there was a small clot present we increased the amount of citrate.

Serum.	Citrate.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	2 c.c.	200 gm.	0.005 c.c.

Death in 2 min. Blood fluid. Lungs emphysematous. Heart beating.

Even without a trace of clotting, we find the serum prepared in this way toxic in the same dose, and post-mortem examination shows a typical lung finding.

We next prepared some hirudinized animals and injected them five minutes after treatment with hirudin. In this case each guinea pig received two minimum lethal doses of the toxic sera.

Dose.	Weight of guinea pig.	Amount of serum per gm. of weight
2.25 c.c.	225 gm.	0.01 c.c.

Typical death in two minutes. No clotting. Lungs emphysematous.

We then used a single minimum lethal dose and also one and one fourth minimum lethal doses, as will be seen below.

Dose.	Weight of guinea pig.	Dose per gm. of weight
0.825 c.c.	165 gm.	0.005 c.c.
1.0 c.c.	160 gm.	0.0062 c.c.

No immediate result. The animals seemed well and were placed in the cage over night. The next morning both were found dead; in each case the blood was fluid, the lungs emphysematous, although not so markedly as in acute anaphylactic shock.

#### INHIBITION OF TOXIC ACTION.

Dilution of a single minimum lethal dose with an equal volume of normal guinea pig serum, and incubation for thirty minutes before injection does not alter the toxicity, but heating to 70° C. for thirty minutes greatly decreases it.

If the assumption that we are removing a protective substance from the serum is correct, then by returning the extracted substance to the serum it should become non-toxic. We have previously shown (1) that by saponifying the ether and chloroform extracts of sera we can recover all the antitrypsin. These protective lipoids are not originally present in the serum as soaps, but inasmuch as it is practically impossible to get the lipoids back into solution in the serum in the fine state of dispersion in which form only they are active, we must saponify them, and in the form of soaps attempt to reintroduce them into the serum. Twenty cubic centimeters of guinea pig blood were placed under chloroform for two hours, the serum was then separated and tested for toxicity.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
0.9 c.c.	180 gm.	0.005 c.c.	Immediate death with intravascular clotting, etc.
0.51 c.c.	170 gm.	0.003 c.c.	Scratches; no symptoms.

The minimum lethal dose was therefore 0.005 of a cubic centimeter per gram of weight. The chloroform extract was mixed with an equal volume of alcohol and water and made strongly alkaline with sodium hydrate. This was heated in the water bath for an hour. The mixture was then acidified, and the fatty acids were

taken up in ether and resaponified by neutralization with alcoholic sodium hydrate. These soaps were now mixed with 1.5 cubic centimeters of the serotoxin and incubated for fifteen minutes.

Dose injected.	Weight of guinea pig.	Dose per gm. of weight.
1.1 c.c.	215 gm.	0.005 c.c.

There were no immediate symptoms and the animal remained well. The toxicity had therefore been completely neutralized. In like manner one can completely neutralize the serotoxin by adding soaps of the unsaturated fatty acids, which, as we have shown (6), have marked antitryptic properties, although they are less active when mixed with serum than when dissolved in salt solution. The serotoxin here used was freshly prepared and autolysis had not occurred, so that preformed toxic split products of proteins (primary proteoses) were probably not present. If on the other hand we use a serotoxin which has been prepared at room temperature by standing for several days, in which there is a marked increase in the total non-coagulable nitrogen, and from which one can isolate toxic proteoses, then the neutralization by the soap is no longer complete, the effect depending of course on the relative amount of the proteins and their toxic split products already formed.

A chloroform serum standing for five days at room temperature caused immediate death in doses of 0.003 of a cubic centimeter per gram of weight. Soap was prepared from serum lipoids and mixed with the dose.

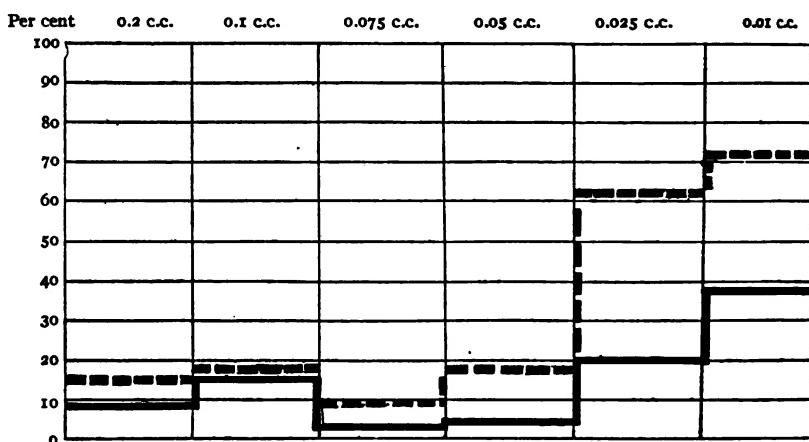
Dose.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	280 gm.	0.0035 c.c.

There were immediate respiratory convulsions, after which the animal recovered for ten minutes; then clonic convulsions occurred at frequent intervals, with death in thirty minutes. In a similar way a dose was prepared by adding 10 mg. of linseed soap (iodin value 110), and in this case, also, while immediate death was prevented, the ultimate result was fatal.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	330 gm.	0.003 c.c.

If the toxicity of the serotoxin depends on the exposure of the proteins, so that they can be easily split to toxic products in the injected animal, then the reduction of the normal antiferments in the injected animal should render the serotoxin more toxic than for a normal animal. A three hour serotoxin (chloroform at

$37^{\circ}$  C.) was used, the minimum lethal dose of which was 0.005 of a cubic centimeter per gram of weight. Four guinea pigs were prepared in the morning by shaving the abdomen and painting with tincture of iodin, so that each animal received about 0.2 of a gram of iodin. In this way one can reduce the serum antitrypsin in an amount shown in text-figure 5, in which the black line represents the



Black line = before treatment (A. M.).  
Dotted line = after being painted with tincture of iodin (P. M.).  
TEXT-FIG. 5. Effect of iodin on antitrypsin of guinea pig.

amount of digestion of casein in the presence of varying amounts of the guinea pig serum drawn in the morning before treatment, and the dotted line represents the digestion in the serum of the same guinea pig in the afternoon. This decrease in antitryptic activity is probably due to the saturation by the iodin of the unsaturated carbon bonds of the fatty acids, as we have stated elsewhere (1).

A sublethal dose was first injected into an iodized guinea pig.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight
0.54 c.c.	180 gm.	0.003 c.c.

Immediate death. Heart blood clotted. Lungs slightly distended. Gall bladder markedly distended.

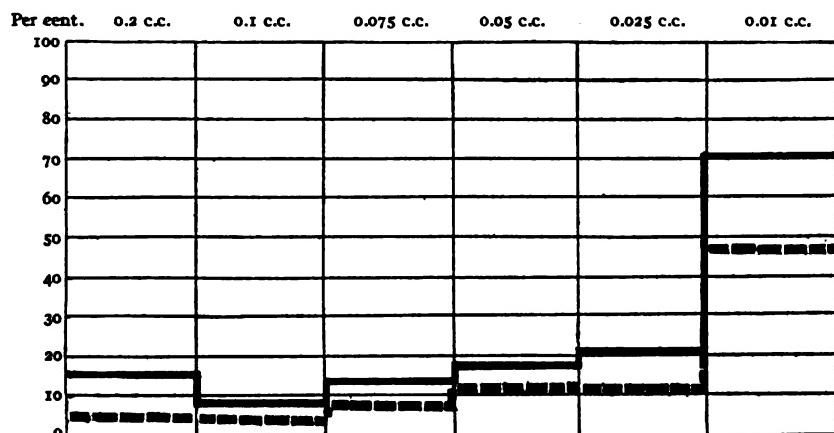
Next a half sublethal dose was given.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight
0.27 c.c.	185 gm.	0.0015 c.c.

The animal was immediately prostrated, sick, with occasional convulsions. Death in thirty minutes. Heart beating. Blood fluid. Lungs slightly distended.

The experiments indicate that the toxicity of the serotoxin is greater for animals in which the antitrypsin has been reduced, thus permitting the ferment normally present to be more active. A single injection of a sublethal dose, given intravenously or intraperitoneally, increases the antitrypsin in the guinea pig; the effect can be demonstrated within twenty-four hours, and persists for at least two weeks. A similar increase has been noted following anaphylaxis (Meyer (7), Pfeiffer (8, 9)). Zinsser (10) has recently noted an increased resistance to a minimum lethal dose of a strong anaphylatoxin prepared from typhoid bacilli, following a single sublethal dose given seven to twelve days previously.

If the serotoxic effects are due to an intravital splitting of the serum protein, then during the period of increased antitrypsin fol-



Black line = original antitrypsin of guinea pig serum drawn on Feb. 24, 1914.  
Dotted line = serum drawn on Feb. 28, 1914.

TEXT-FIG. 6. Increase of antitrypsin following a single injection of a sublethal dose of serotoxin.

lowing a sublethal dose there should be an increased resistance to the serotoxin. The effect of a sublethal dose on the guinea pig antitrypsin is shown in text-figure 6. The black line indicates the percentage of casein digestion as influenced by the normal guinea pig serum drawn on February 24, 1914. A sublethal dose of serotoxin was given intraperitoneally on the same day. A second

sample of blood was drawn on February 28, and is shown in the dotted line. As will be seen, there is a well marked increase in inhibiting power.

The increased resistance to serotoxin is shown in the following experiment (February 24).

Dose of serotoxin.	Weight of guinea pig.	Dose per gm. of weight.
1.9 c.c.	190 gm.	0.01 c.c.
Guinea pig sick and prostrated. Complete recovery.		

The following day the guinea pig received 0.006 of a cubic centimeter, per gram of weight, of a serotoxin preparation of which the minimum lethal dose was 0.005 of a cubic centimeter. There were no symptoms. After several days following the original sublethal dose we have secured animals that are resistant to two minimum lethal doses.

#### EXPERIMENTS WITH PURIFIED HORSE SERUM ALBUMEN.

In order to compare the results which we had so far obtained with a pure protein intoxication we prepared some horse serum albumen in the following manner: The globulins of the serum were thrown out of solution by half saturation with ammonium sulphate, and after filtration, the albumens were removed from the filtrate by precipitation with acetic acid. This precipitate was redissolved in water made slightly alkaline with sodium carbonate, treated with an equal amount of a saturated solution of ammonium sulphate, filtered, and the filtrate again treated with acetic acid. The final precipitate was dialyzed against running water until free of acid and ammonium sulphate, and was then evaporated to dryness. It was made up in a 1 per cent. solution in normal saline. Of this solution a dose of 0.02 of a cubic centimeter per gram of weight was not toxic for the guinea pig. On testing its antitryptic property it was found that two cubic centimeters of such a solution inhibited 86 per cent. of tryptic digestion, and on digesting two cubic centimeters with a strong trypsin solution for forty-five minutes, only 0.1 of a milligram of non-coagulable nitrogen was obtained. If we now extract the solution by mixing with chloroform at 37° C. for two hours we find the toxicity well marked in the dose which was not at all toxic for the unextracted serum.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4 c.c.	200 gm.	0.02 c.c.	Immediate death. Heart blood clotted. Lungs distended.
2 c.c.	190 gm.	0.01 c.c.	No effect.

After extraction for twenty-four hours the toxicity had still increased.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.	Result.
2.4 c.c.	240 gm.	0.01 c.c.	Killed in 2 min. Heart blood clotted.
0.9 c.c.	180 gm.	0.005 c.c.	No effect.

The last dose, which was sublethal, was now injected into an iodized guinea pig. The method of preparing the guinea pig has been already described.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1.1 c.c.	220 gm.	0.005 c.c.

Immediate death. Heart blood fluid. Lungs distended. Gall bladder distended.

As with the serotoxin, there was a lessened resistance to the toxic effect in the iodized guinea pig. When the antitryptic effect of the extracted (twenty-four hour) horse serum solution was tested, it was found that it now inhibited only 33 per cent. of casein digestion, and was itself digested by trypsin much more easily (0.35 of a milligram for 2 cubic centimeters of solution). After extraction for seventy-two hours the toxicity had increased so that a dose of 0.005 of a cubic centimeter per gram of weight caused marked toxic symptoms but did not kill. At this time the serum albumen was no longer antitryptic. Inasmuch as there are no ferment present in the serum albumen solution, and no trace of autolysis can be obtained when such solutions are incubated, there is no preformation of toxic split products, and whatever increase in toxicity occurs must be due to the increased availability of the serum albumen for attack by the ferment of the injected animal.

In the serotoxin we have a somewhat different condition due to the presence of quite active proteolytic ferment. It would therefore seem reasonable that the ferment would attack the serum proteins as soon as the antitrypsin had been completely removed, and

produce toxic split products,—primary proteoses. This condition actually obtains. If one fractionates a serotoxin which has been under chloroform at 37° C. for only two hours, only faint traces, or no evidence at all, of primary proteoses can be obtained. From twenty-four hour serotoxin we have obtained toxic proteoses, as is shown in the following experiment.

Ten cubic centimeters of normal guinea pig serum were mixed with chloroform and incubated for twenty-four hours. The chloroform was then removed, the native protein removed from the serum by the heat and acid method, and the filtrate made faintly alkaline to throw out the acid albuminates, and again filtered through kaolin. The primary proteoses were then precipitated by half saturation with ammonium sulphate. These were removed, dialyzed until free from all traces of ammonium sulphate, and then made up to the original volumes. When injected intravenously in 0.1 per cent. salt solution the primary proteoses are toxic in a dose of about 0.01 cubic centimeter per gram of weight. When so prepared the toxicity is unaltered by boiling.

#### DISCUSSION.

In the preliminary experiments we observed the marked similarity to anaphylactic death which our animals showed on the injection of the serotoxin, and later, while working with the rapidly prepared chloroform sera, the paradoxical similarity to cytotoxic death—immediate coagulation—was noted. In view of the fact that the usual method of death is intravascular coagulation, we have used the term serotoxin to designate the entire toxic effect of the extracted sera, although we wish to emphasize the importance of the protein cleavage phenomena.

The toxicity of organ extracts first noted by Brieger and Uhlenhuth (11), was shown by Blaizot (12), Gley (13), and by Dold and Ogata (14) to depend on the intravascular coagulation, and could be rendered completely inert by the treatment of animals with hirudin. Ichikawa (15) has recently shown that sodium citrate will also render a toxic dose non-toxic. Serotoxin differs fundamentally from the cytotoxin in that the toxic effects persist when coagulation is prevented by hirudin or citrate. The toxicity is due to

factors quite apart from the altered coagulation balance, although we believe that it will be found that the unsaturated lipoids play an important part in the mechanism of coagulation and its prevention.

From our experiments we believe that we are justified in concluding that the exposed homologous serum proteins are capable of being split when injected into an animal, and produce the observed toxic effect. In many of our experiments the exact picture of acute anaphylactic death was observed; in sublethal doses a marked fall in temperature and extreme prostration were noted, the latter being most marked in sublethal doses of sera which had been permitted to autolyze. The toxicity of serum protein is well illustrated in the experiment with purified horse serum albumen which was not toxic in doses of 0.02 of a cubic centimeter of a 1 per cent. solution per gram of weight, but when extracted with chloroform it gradually became more and more toxic until a final dose of 0.005 of a cubic centimeter per gram of weight (or 0.00005 of a gram of serum albumen per gram of weight) was sufficient to cause marked toxic effects. The length of time required to extract the lipoids from this solution indicates how intimate the relation of the lipoid to the serum albumen complex must be.

That the exposed serum proteins are the substances which produce *in vivo* the toxic effects when split, is shown by the fact that in the rapidly prepared serotoxin no increase in non-coagulable nitrogen is demonstrable, and that no toxic split products (primary proteoses) can be isolated by fractioning the serum proteins at this time. The antiферments having once been extracted, there is of course no further impediment to ferment action, such sera become actively proteolytic, as was first shown by Delezenne and Pozerski (16), and undergo autolysis as shown by the marked increase in the non-coagulable nitrogen. Under these conditions we have always been able to isolate preformed toxic split products. The effect of the soaps, too, in not completely neutralizing the serotoxin at this period is confirmatory evidence, since the soaps act by preventing the splitting of native proteins (true antitryptic effect, rather than anti-ereptic). With continual autolysis we should expect lessened toxicity, and this is found to be the case. That the

normal inhibitory mechanism of the serum markedly influences the intravital splitting, as it does in the test-tube, is shown by the great increase of toxicity of the serotoxin for animals with lessened anti-ferments and by the greater resistance of animals with increased anti-ferments. We believe that the effect of the iodin is due to a saturation of the unsaturated bonds of the fatty acids of the lipoids, rendering the substituted product less active as an anti-tryptic agent. The effect of a single sublethal dose of serotoxin in increasing the anti-ferment can possibly be explained by a lowered oxidation of the organism due to the toxin, with a resulting lowering of the physiological oxidation of these unsaturated compounds, or it may be due to a breaking up by the toxic action, of protein-lipoid combinations with a resulting increase in the lipoidal elements in the blood. From our studies we are even more firmly convinced of the biological importance of the protein-lipoid combination in the organism, and the correlated necessity of according to lipolytic action greater emphasis in the part of the adjunct which breaks up these combinations before actual proteolytic changes can take place. The small amount of purified horse serum albumen (after complete chloroform extraction) required to kill a non-sensitized animal is significant from this point of view.

A large number of investigators, notably Vaughan (17), Friedberger (18), and Pfeiffer (9), have, during the past few years, emphasized the importance of the split products of proteins in the production of the toxic effects in the various infections, but the basic idea has always been held that the substrate which was acted upon was the foreign protein concerned, and that the homologous proteins might be neglected, although the work of Keysser and Wassermann (19), Doerr (20), Bordet (21), and Ritz and Sachs (22) indicate that adsorption phenomena and colloidal changes might render homologous sera toxic. The idea, however, that the adsorbed substances were the anti-ferments, or protective agents, has not been expressed, and no efforts have been made to remove such anti-ferments, possibly because of the unfortunate view that the anti-ferments represented immune bodies or split products of the protein molecule.

From a clinical point of view we are inclined to believe that the

proof of the toxicity of exposed serum proteins may be of importance. Longcope (2) has recently shown that repeated injections of heterologous proteins can cause marked nephritic changes. The profound prostration shown by leukemics in the terminal stages when the serum ferments are known to increase greatly, so that such sera may become proteolytic, suggests the profound prostration observed in experimental animals injected with sublethal doses of autolyzed sera. Further experiments are now in progress to determine the results from chronic sero-intoxication.

#### CONCLUSIONS.

1. Sera from which the protective lipoids (unsaturated fatty acids) have been removed are toxic for the homologous animal.
2. The toxicity is due to three factors: (a) an alteration in the mechanism of coagulation, with resulting intravascular coagulation; (b) the exposure of the native serum proteins; (c) the formation of toxic split products (primary proteoses) by autolysis.
3. A definite maximum of toxicity can be determined, with a final stage of atoxicity due to continued autolysis.
4. Hirudin and sodium citrate do not protect animals.
5. Heating to  $70^{\circ}$  C. destroys, or greatly lessens, the toxicity of the serotoxin, although the isolated proteoses are toxic after boiling.
6. The return of the extracted lipoids (saponified) neutralizes the toxicity.
7. Unsaturated soaps also neutralize the toxicity.
8. Sublethal doses produce extreme prostration, marked fall in body temperature, no eosinophilia, and an increase of antitrypsin.
9. Sublethal doses of rapidly prepared chloroform sera cause a decrease in coagulation time; sublethal doses of autolyzed sera cause an increase in coagulation time.
10. Previously injected animals are more resistant (increased antiferments).
11. Iodized animals are less resistant (decreased antiferments).

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FURTHER OBSERVATIONS ON THE GROWTH OF BACTERIA ON MEDIA CONTAINING VARIOUS ANILIN DYES, WITH SPECIAL REFERENCE TO AN ENRICHMENT METHOD FOR TYPHOID AND PARATYPHOID BACILLI.\*

By CHARLES KRUMWIEDE, JR., AND JOSEPHINE S. PRATT.

(*From the Research Laboratory of the Department of Health, New York.*)

In a previous communication (1) we reported the results of a series of observations on the growth of thirty varieties of bacteria on media containing various dyes. We have continued this work using the same series of cultures and forty samples of dyes. The methods employed have been the same. Green dyes are widely used in special media for typhoid and paratyphoid isolation. This led us to include many of these dyes in our present work, in the hope that some might be more selective than those in use. If this were the case, their application to plate and fluid media would be of great value.

In general, the results have been similar to those with other dyes, especially gentian violet and its allies (table I). The inhibition of growth has been most evident among the Gram-positive bacteria, the Gram-negative bacteria as a rule growing freely on the dyed agar.

Certain exceptions are evident in the table. The cholera strain employed was less resistant to the action of green dyes than the allied vibrios. To determine whether this was of differential value, forty cultures of cholera and other vibrios were tried on agar containing *Bittermandelölgrün*, the dye showing the greatest differences. A variable restraining action was noted, but there were no differences according to types.

The diphtheria bacillus and its allies have given apparent differences and irregularities. This is due to the tendency of these cultures to produce flakes which, when inoculated on the agar, produce a limited growth, whereas the separate bacilli fail to grow.

\* Received for publication, February 18, 1914.

TABLE I.1, 2

Bacteria tested.	Brilliant green crystals 266 (Bayer).	Brilliant green.	Anilin green.	Solid green.	Victoria green crystals 4-83 (Bayer).	Smaragd green.	China green.	Malachite green.	Bitter-mandelölgrün.	Victoria blue 4 R.	Malachite green (Höchst).
B. coli communis . . . . .	+	*	-	-	+	*	-	+	+	+	+
B. coli communior . . . . .	+	*	-	-	+	*	-	+	+	+	+
Friedländer's bacillus . . . . .	+	*	-	-	+	*	-	+	+	+	+
B. lactis aerogenes . . . . .	+	*	-	-	+	*	-	+	+	+	+
B. ozænæ . . . . .	-	-	-	-	-	-	-	-	-	-	-
B. pullorum . . . . .	-	+	-	-	-	-	-	-	-	-	-

<sup>1</sup> In the tables + = growth like control; # = restrained growth; X = markedly restrained growth; -- = no growth;

\* = some growth developed later; § = several colonies.

<sup>2</sup> In addition to the dyes given in the table, the following were also tested: alkali green, *Säurealizaringrün*, *Lichgrün F. S.*, *Brillantblau (extra grünlich)*, naphthol green B, *Echtgrün*, *Säuregrün*, alizarin green B, methyl blue, trypan blue, anilin blue (water-soluble), China blue, anilin blue-black, *Blaukwarz*, and *Wasserblau*. In all the dilutions employed growth was as vigorous as on the control. Where not otherwise stated, all the dyes employed were obtained from Grüber.

TABLE I.—Concluded.

Bacteria tested.		Gram-positive		Gram-negative	
		Methy-	Chlor-	Methy-	Gram-
		ene green.	drin blue,	ene green.	negative
Staphylococcus aureus	+	-	-	-	-
Streptococcus (green)	+	-	-	-	-
Streptococcus (hemolytic)	+	-	-	-	-
Streptococcus mucosus capsulatus	+	-	-	-	-
Pseudomoccus	+	-	-	-	-
B. diphtheriae	*	-	-	-	-
Diphtheroid bacilli (Cameron)	*	-	-	-	-
Diphtheroid bacilli (Cunningham)	*	-	-	-	-
B. hoffmanni	*	-	-	-	-
B. subtilis	*	-	-	-	-
B. lactimorbi	*	-	-	-	-
B. anthracis	*	-	-	-	-
Cholera	+	-	-	-	-
Vibrio (indol-positive)	+	-	-	-	-
Vibrio (indol-negative)	+	-	-	-	-
B. proteus	+	-	-	-	-
B. procyanus	+	-	-	-	-
B. dysenteriae (Shiga)	+	-	-	-	-
B. dysenteriae (Flexner)	+	-	-	-	-
B. dysenteriae (Mt. Desert)	+	-	-	-	-
B. typhoaeus	+	-	-	-	-
B. paratyphosus A	+	-	-	-	-
B. paratyphosus B	+	-	-	-	-
B. enteritidis (Gaertner)	+	-	-	-	-
B. coli communis	+	-	-	-	-
B. coli communis	+	-	-	-	-
Friedländer's bacillus	+	-	-	-	-
B. lactis aerogenes	+	-	-	-	-
B. osmanii	+	-	-	-	-
B. piliformis	+	-	-	-	-

With one exception the results obtained have failed to show variations which could be used for practical differentiation. They are given, however, as similar detailed observations are not available in the literature.

The observations on the green dyes show marked variations in the resistance of members of the typhoid-paratyphoid-colon group. In general the paratyphoid-enteritidis types are highly resistant, the typhoid less so, but somewhat more resistant than the coli types. Various green dyes (2) have been employed in media to suppress the colon types found in feces. Loeffler employed malachite green agar, Werbitzki China green, and Conradi brilliant green agar, adding picric acid which reduces the activity of the dye. The media have been of some use, especially for preliminary plating for enrichment, for paratyphoid more than for typhoid. On these media the difference in resistance of the typhoid and colon types was slight. If very few typhoid bacilli were present or if less resistant than the average, failure resulted. Peabody and Pratt and others attempted to use malachite green in fluid media as an enrichment medium.

Of the dyes we tried, ten revealed variations among the typhoid-colon group. A large number of cultures of the group were obtained<sup>3</sup> and grouped according to their sugar fermentations, and tried on agar containing some of these dyes. This was done to determine whether one dye showed wider differences than another and as a preliminary to the application of the results already obtained, to a selective plating medium, or a fluid enrichment medium. Agar is an easier medium to employ as an index, and was therefore used, as we had found that the results on agar could be duplicated in broth with slight variations in dilutions. To reveal slight differences in reaction, a small number of bacteria were inoculated. A loopful of a broth culture was carried to a second tube and loop inoculations were made from this (table II).

The results show that the different dyes possess a similar differential action. This difference, however, appears at different dilutions according to the dye employed. At the appropriate dilutions no one dye is perceptibly more differential than another.

<sup>3</sup> We are indebted to Dr. Torrey and Prof. C.-E. A. Winslow for some of the cultures employed.

*Observations on the Growth of Bacteria.*

TABLE III.4.5

Type tested.	No. of strains.	Anilin green.	Bright green (Griibler).	Bright green (Bayer).	Smarged green.	Solid green.	Victoria green (Bayer).	China green.
B. alkaligenes	2	-	-	-	-	-	X + # + + + + X : X : : : - + * + -	: X + # + + + + + X : X : : : - + * + -
B. typhosus	3	+	-	-	-	-	: X + # + + + + + + : + : : : - + * + -	: X + # + + + + + + : + : : : - + * + -
B. paratyphosus, A	6	+	-	-	-	-	: * + - + + + + + : + : : : - + * + -	: + + + + + + + + + : + : : : - + * + -
B. paratyphosus, B	4	+	-	-	-	-	: X + # + + + + + + : + : : : - + * + -	: * + - + + + + + + : + : : : - + * + -
B. enteritidis	2	-	-	-	-	-	-	-
Intermediate types . . . . .	{ 4	+	+	+	+	+	-	-
B. acidi lactici	6	+	-	† or -	-	-	-	-
B. coli communis	3	+	+	+	+	+	-	-
B. coli communior	6	+	+	+	+	+	-	-
B. aerogenes types . . . . .	6	+	-	†	†	+	-	-

\* Where two sets of observations are given they are the extremes on that type of culture.

— = no fermentation; + = acid production; † = production of acid and gas; .. = no observations. Other symbols are as in preceding table.

Five cultures were not included in the preceding table. These are the cultures used by Churchman and Michael (3). We have taken their observations on the growth of these cultures on gentian violet agar and their interagglutinations, and added our observations of the sugar fermentations of the same cultures and of the growth on media containing green dyes. Table III shows the possible application of dye media to the differentiation of some members of the enteritidis group. The question that immediately arises is: Has the reaction to dyes any relation to their source or pathogenicity? For instance, two cultures, E 25 and E 26, were isolated from a drainage canal by Jordan. Unfortunately the source of the other cultures is not available. If the strains which did not grow came from human beings suffering from infection by these organisms, there will be times when the application of these dyes for enrichment methods will fail.

TABLE III.

Organism.	Growth on gentian violet agar.	Growth on agar, or in broth with green dyes. <sup>a</sup>	Glucose.	Dulcite.	Summary of agglutination reactions.			
					Sera.			
					E 18.	E 25.	E 26.	E J.H.H. same as E 234.
E 18 (67).....	+	+	†	†	+++	o	o	++
E 25 (68).....	+	- <sup>b</sup>	†	-	o	+++	o	o
E 26 (14).....	+	- <sup>b</sup>	†	†	o	o	+++	o
E 132 (70).....	+	+	†	†	+++	o	o	++
E 234 (64).....	-	- <sup>b</sup>	†	†	++	o	o	+++

Experiments were then begun with fluid and solid media for differential enrichment. The use of solid media was not as successful as fluid media. As we had not found any one dye more selective than another and because of two reports giving results similar to our own which appeared at this time, we continued to work with brilliant green.

Torrey (4) found that by the use of Grüber's brilliant green in fluid media he could enrich the paratyphoid-enteritidis group and restrain the common fecal bacteria. Browning, Gilmour, and Mackie (5) were able, by the use of Bayer's brilliant green in fluid

<sup>a</sup> Dyes given in table II.

<sup>b</sup> Very slight delayed growth on agar containing 1:500,000 of some of the dyes.

media, to isolate typhoid or paratyphoid bacilli from stools, where direct plating was unsuccessful.

Preliminary tests were made to determine what medium to use. Peptone and meat extract broth with and without glucose and containing the dye in various dilutions were tried. Counts were made of the number of paratyphoid bacilli inoculated and the number per loop of the broth after incubation. The glucose extract broth gave the most abundant growth. This medium was therefore used in our subsequent work. For all our work we have used media neutral to phenolphthalein.

Some experiments had shown that slight changes would lower the threshold of growth for the typhoid bacillus but make little change in the restraining action of the dye towards the coli types; for instance, small additions of proteid substances change the action of the dye. A test was made, therefore, of the influence of added feces as a preliminary to selecting dilutions for work with stools. Typhoid bacilli, being less resistant, were used for this test. The results with Bayer's brilliant green (table IV) show that about one third of the activity of the dye is lost.

TABLE IV.

	No. per loop after 18 hours' incubation.		
	500,000.	400,000.	300,000.
Typhoid (213 bacilli inoculated).....	218	0	0
Typhoid + 0.1 c.c. of boiled feces (213 typhoid bacilli inoculated).....	+++ <sup>a</sup>	+++	+++
0.1 c.c. of boiled feces (control).....	0	0	0

As a further index to the selection of dilutions, the possible variation of different strains to the dye and to different types of the dye was determined. Further strains were tried in broth containing Bayer's brilliant green. The number of bacilli inoculated ranged from one to seventy. No growth, as determined by plating a loopful of the media, occurred in dilutions after and including 1 to 400,000. In 1 to 500,000 two showed multiplication. A loop plated from these tubes showed only 24 and 500 colonies. Repeating this with six strains with Grüber's dye gave essentially similar results.

<sup>a</sup> Like plain broth control.

*Results of Examination of Stools.*

Name of case.	Direct plates (Conradi).	Couradti plates after enrichment in broth containing brilliant green.	Source of dye.
R.	Less than 1% typhoid	1:500,000 10% typhoid <sup>10</sup>	Over 50% typhoid; many bluish colonies not typhoid 1:400,000
M.	Negative (overgrowth by red colonies)	60% typhoid	75% typhoid Overgrown by aerogenes types (lower dilutions the same)
O.	30% typhoid	Overgrown by aerogenes types	Many hundred typhoid colonies Overgrown by aerogenes types; typhoid isolated from plate (lower dilutions gave aerogenes types only)
M.	7% typhoid	90% typhoid	95% typhoid Grubler.
M.	7% typhoid	Many typhoid colonies where plate is clear of heavy red growth; plate red from diffusion	Clear parts of plate peppered with typhoid colonies; heavy red growth with diffusion of acid through plate Moderate number of typhoid colonies; plate overgrown by aerogenes types
O.	50% typhoid	Very many typhoid colonies where plate is free from over-growth of aerogenes types	Few typhoid; practically overgrown by aerogenes types Moderate number of typhoid colonies; plate overgrown by aerogenes types
O.	50% typhoid	Very many typhoid colonies where plate is free from over-growth of aerogenes types	Few typhoid; practically overgrown by aerogenes types Plate overgrown by mucous types
R.	Five typhoid colonies or three plates	Confluent growth, red margin and bluish center, many fine bluish colonies between confluent lactose-fermenting aerogenes types.	Confluent growth, red margin and bluish center, many fine bluish colonies between confluent lactose-fermenting aerogenes types; impossible to identify typhoid by direct agglutination from plate; fishings gave slow
R.	Five typhoid colonies or three plates	Confluent growth, red margin and bluish center, many fine bluish colonies between confluent lactose-fermenting aerogenes types.	Confluent growth from plate or by fishing onto Russell's medium with subsequent agglutination. impossible to identify typhoid by direct agglutinations of growth from plate; fishings gave slow

\* Eight other stools were examined and were negative both by direct plating and after growth in dye broth.

<sup>10</sup> Verified by direct agglutinations of growth from plate or by fishing onto Russell's medium with subsequent agglutination.

Two strains, however, showed slight multiplication at the 1 to 400,000 dilution.

Because of these results dilutions of 1 to 500,000 to 1 to 100,000 were tried in testing the stools from typhoid carriers. The stools were from three cases and were examined on two occasions, once with Bayer's dye only, and once with both makes of dye. The broth was used in ten cubic centimeter amounts, and 0.1 of a cubic centimeter of thinned feces was added to each tube. The results are given in table V.

That we can enrich typhoid by this method is evident. The results confirm those of Browning, Gilmour, and Mackie. We also agree with their suggestion to use graded dilutions. When we add feces we are introducing a variable factor and the typhoid strains also vary somewhat in their resistance to the dye. Success depends on the right adjustment. The value of the method must rest on the results of routine examinations over a period of time. From our results we would suggest the use of the three dilutions as given, and the addition of 0.1 of a cubic centimeter of thinned feces. The overgrowth of some of the plates suggests the advisability of streaking two plates from each tube, without burning the wire between the plates. Unsuccessful results from overgrowth are apparently due most frequently to *Bacillus aerogenes* types, which are extremely resistant to the dye and usurp the plate where seeding is too heavy.

The range of variability of the available paratyphoid-enteritidis strains was determined. Table VI gives the results.

The results show a wide range of variability. Certain members of this group, as already noted, failed completely to grow. How far such variations will be present in strains in human feces will be shown only by the results of routine examinations.

The results with our cultures make us doubt the advisability of using only one dilution,—1 to 6,600, as advised by Torrey. Although he grades the amount of feces, we doubt whether this would reduce the activity of the dye sufficiently to allow the growth of the less resistant types, assuming that such strains will occur in naturally infected feces. Furthermore, such low dilutions may not weed out the more resistant fecal types of bacteria any more than would the

TABLE VI.<sup>11</sup>

Culture and type.		No. inoculated.	Brilliant green (Bayer).		No. inoculated.	Brilliant green (Grübler).		
			No. per loop after 18 hours.			No. per loop after 18 hours.		
			$\times 100,000$	$\times 10,000$		$\times 100,000$	$\times 10,000$	
Paratyphoid, A	3	53	+	+	92	++	1320	
	54	228	++	242	300	+	99	
	56	364	++	++	496	++	++	
	57	392	++	+	248	+	85	
	58	314	++	+	640	++	++	
	60	304	++	++	47	++	++	
	62	59	++	o	112	o <sup>12</sup>	o	
	63	234	2 <sup>12</sup>	o	400	2 <sup>12</sup>	o	
	66	200	++	++	240	++	++	
Paratyphoid, B	52	600	++	+	412	+	214	
	53	282	++	++	55	++	++	
	59	85	++	o	18	o <sup>11</sup>	o	
	61	140	++	+	228	++	++	
	65	142	++	+	160	++	++	
Enteritidis	4	384	4 <sup>12</sup>	o	668	552 <sup>12</sup>	129	
	47	270	++	++	160	++	+	
	67	360	++	++	352	++	++	
	70	298	++	++	480	++	+	

higher dilutions. Some aerogenes types are more resistant than the most resistant paratyphoid-enteritidis strains. In fact two of Torrey's strains showed decidedly less resistance than the others, and acted more like some of our strains. His results with feces of dogs artificially infected prove the value of the method, but are no index to the correct dilutions to employ. The strains used for feeding are not stated, but were undoubtedly fully resistant to the dye. This proves nothing as to the results to be expected in routine examinations where strains may vary.

As no naturally infected stools were at our disposal, we did not continue work in this direction. From our results with cultures, graded dilutions seem to us to be indicated in stool examinations for paratyphoid and enteritidis. Taking into consideration the fact that the feces itself will lower the activity of the dye, a series of dilutions ranging from 1 to 100,000 to 1 to 500,000 will probably cover the variations we have noted, with the exception of those

<sup>11</sup> ++ = closely crowded colonies, like control; + | = closely crowded colonies, less than control; + = closely crowded colonies, less than preceding.

<sup>12</sup> Quantitatively similar restraint in 1:200,000.

strains which were completely inhibited, and restrain the fecal organisms that can be restrained.

Our results in the attempt to apply these results to a solid medium have been only partially successful. The green dyes can be used as by Conradi, but this has the disadvantage of the absence of an indicator for lactose fermentors. The use of sodium sulphite with the green dye or some other added dye, as fuchsin, has the disadvantage that the sulphite lowers the activity of the dye and a balance is obtained with difficulty. The attempt to find an indicator which would be sharp but not influence the activity of the green dye has so far been unsuccessful.

#### CONCLUSIONS.

Several green dyes show a marked selective action for members of the typhoid-paratyphoid-colon group. This can be used for the enrichment of typhoid and paratyphoid bacilli present in feces. Forty dyes were tested with thirty strains covering all types of pathogenic bacteria. In general the dyes restrained the growth of the Gram-positive bacteria but had no effect on the growth of the Gram-negative group.

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## FACTORS OF RESISTANCE TO HETEROPLASTIC TISSUE-GRAFTING.

### STUDIES IN TISSUE SPECIFICITY. III.\*

By JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

#### PLATES 56 TO 60.

Previous observations have tended to show conclusively that tissues cannot be transplanted from one species to another, even though these be closely related. Two theories have been brought forward to explain this failure in heteroplastic grafting. The two schools are still at variance and neither has been able to produce evidence conclusive enough to convince the other. The first and most prominent theory is that of Ehrlich, termed athrepsia.<sup>1</sup> The experimental foundation for this hypothesis is the so called zigzag transplantation of tumors between rats and mice. It was observed that a mouse tumor when grafted into a rat, or *vice versa*, would survive and proliferate for six to eight days, but would later fail rapidly and be absorbed. If, however, the mouse tumor was removed during the proliferating stage and reinoculated into a mouse it continued to grow actively. After a period of six or eight days' active growth in the mouse it could again be grafted into a rat. This zigzag grafting could be carried on indefinitely with no apparent effect on the tumor tissue or in lessening its activity of growth. The interpretation, suggested by Ehrlich, is that each species provides its tissues with a specific food substance (X) which is necessary for its maintenance and growth. The temporary survival of the mouse tissue in the rat is due to the amount of this specific food carried over with the graft. When this is exhausted the graft dies unless returned to its native species, where it will accumulate a fresh supply of the specific food and again be able to survive for a time in a foreign species.

\* Received for publication, March 24, 1914.

<sup>1</sup> Ehrlich, P., *Arb. a. d. k. Inst. f. exper. Therap.*, 1906, No. 1, 84.

The chief opponent of this theory is Bashford<sup>2</sup> who rests his objection on the findings in an experiment in which rats were inoculated a second time with mouse tumor. Under these conditions the second graft, although containing an equal amount of the hypothetical food substance, would survive only two to three days. From this fact he concludes that there is an active immunity developed against the cancer cell as a foreign proteid. The time of survival of the first graft he considers the time required for the development of the active immunity. Bashford<sup>3</sup> claims that the immunity to homoplastic grafting is an entirely different process and that it depends entirely on the blood vessel and stroma reactions. The merits of the two theories will not be discussed; they are quoted to give an idea of the present views on the subject.

#### LYMPHOCYTIC REACTION IN RELATION TO TISSUE GRAFTS IN IMMUNE ANIMALS.

The occurrence of a lymphocytic reaction around tissue grafts in immune animals has been pointed out by numerous observers<sup>4</sup> and arises whatever the type or source of the animal's immunity. The immune states are: the natural immunity possessed by an animal individually, or because of variety of species; the acquired immunity which is present in animals that have recovered from a primary or implanted tumor; and finally the so called artificial immunity which can be induced by one of several procedures. The small round cell or lymphocytic infiltration is present when there is healing in a spontaneous tumor, and is seen around the edge of slowly growing cancers in man. The importance of these cells in the immunity reaction to tissue grafts would seem evident, yet they have received scant attention.

#### HETEROPLASTIC GRAFTING IN A NON-RESISTANT ORGANISM, THE CHICK EMBRYO.

In a previous communication<sup>5</sup> it has been pointed out that the avian embryo has no defensive mechanism against the growth of

<sup>2</sup> Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

<sup>3</sup> Bashford, E. F., and Russell, B., *Lancet*, 1910, i, 782.

<sup>4</sup> For the literature see Da Fano, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, v, 1.

<sup>5</sup> Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1912, lix, 874.

tissues of a foreign species. The tumor tissue of a rat, for instance, by transference from embryo to embryo could be kept growing in the chick for an indefinite period.<sup>6</sup> The rat tissue underwent no marked change during its long sojourn in the chick embryo, as was shown by the fact that at any time during this period it could be replanted successfully into its native species but was promptly disintegrated when grafted into the adult chicken. It was later shown<sup>7</sup> that a defensive mechanism developed rapidly in the embryo at about the time of hatching, quickly destroying any foreign tissue that might be present. The foreign species tissue growing in the embryo shows a total absence of a round cell reaction. The lymphoid cells around the graft first become evident at about the time that the defensive mechanism begins to show its effect. Other than this there is no great change in the embryo to account for this sudden development from a susceptible to a highly resistant organism. If this change is the result of the sudden activity of a tissue or organ formerly quiescent, it should be possible to provide the embryo with this necessary tissue or organ by grafting various adult tissues into the chick embryo.<sup>8</sup>

#### ACTION, IN VITRO, OF TISSUES IN HOMOLOGOUS PLASMA ON THE GROWTH OF HETEROLOGOUS TISSUE.

Lambert and Hanes<sup>9</sup> have shown that the tissues of one species are capable of growth in the plasma of certain other species. As a preliminary step to the experiment suggested above an attempt was first made to determine the interaction of tissues *in vitro*. Bits of a rapidly growing rat sarcoma were placed in drops of chicken plasma and to these were added in series bits of various adult chicken tissues. The cultures were mounted in hollow slides according to the well known method. The rat tissue in chicken plasma grew remarkably well and was not affected by adult chicken connective tissue, kidney, or liver in close proximity. When, however, a bit of adult chicken spleen was growing in the same drop

<sup>6</sup> Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.

<sup>7</sup> Murphy, Jas. B., *idem*, 1914, xix, 181.

<sup>8</sup> Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1914, lxii, 199.

<sup>9</sup> Lambert, R. A., and Hanes, F. M., *Jour. Exper. Med.*, 1911, xiv, 129.

of plasma there was practically a total inhibition of the growth of the rat sarcoma. The only other tissue showing a similar effect was the bone marrow, which caused definite retardation, but not so marked an inhibition as that brought about by the spleen.

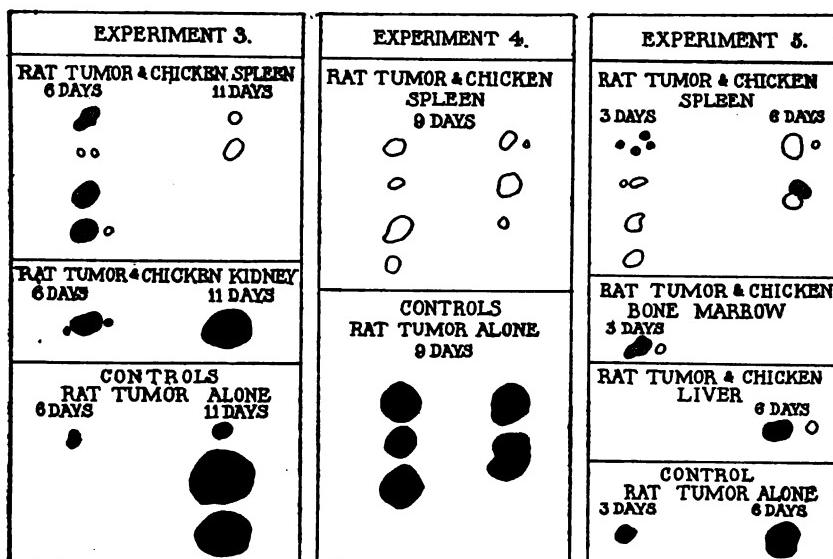
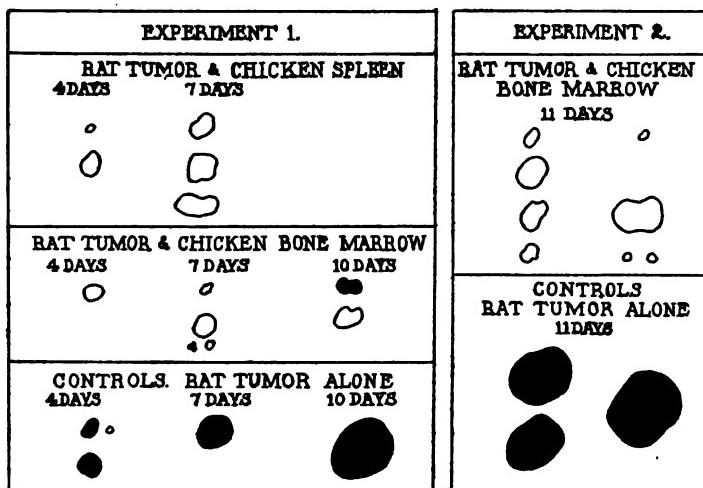
#### THE EFFECT OF ADULT CHICKEN TISSUE GRAFTS ON THE HETEROPLASTIC GRAFTING IN THE EMBRYO.

Since it is possible to graft various adult tissues into the embryo<sup>10</sup> the above experiment was repeated *in vivo*.

In the first series, comprising twenty experiments and over 150 embryos, grafts of rat sarcoma and bits of adult chicken tissues were placed side by side in the outer membrane of seven day chick embryos, according to the method previously described. The adult chicken tissues used were spleen, kidney, liver, bone marrow, and connective tissue. The eggs were returned to the incubator, and at intervals up to the eighteenth day of incubation part of each lot was opened and the grafts were removed for microscopic examination. Text-figure 1 shows the results in a few of these experiments. In the instances where adult chicken kidney and rat sarcoma were inoculated together the resultant tumors were as large as the controls of rat tumor alone; that is, they generally measured from one to two centimeters. Microscopic examination showed the rat cells in active proliferation, with the kidney tubules, also in good condition, scattered through the tumor mass or in a clump at its edge (figure 1). Chicken liver grafts generally caused a widespread necrosis of the membranes of the chick. When bits of the liver graft survived they were found to consist of a few scattered bile capillaries. If the rat tissue graft escaped the necrosis it was found to be in as active growth as the controls. Connective tissue of the adult chicken had no effect on the rat tumor cells in the embryo, although the connective tissue itself grew well.

The striking result was obtained when grafts of adult chicken spleen were inoculated with the rat tumor. The resulting tumors instead of being well rounded, greyish, and semitranslucent were flat, often mottled, yellowish, and opaque. Microscopic examination of specimens removed after three or four days showed the

✓<sup>10</sup> Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.



TEXT-FIG. 1. This chart shows in silhouette the results of simultaneous inoculation of rat sarcoma and a graft of adult chicken tissue into the outer membrane of chick embryos. The unshaded nodules were found on microscopic examination to be made up of the adult chicken tissue and reactive tissue, but showed no surviving rat cells. The shaded nodules were found to have a few rat cells embedded in a mass of reactive tissue (figures 2, 3, and 6). The black represents tumors in active growth, with no sign of defensive reaction.

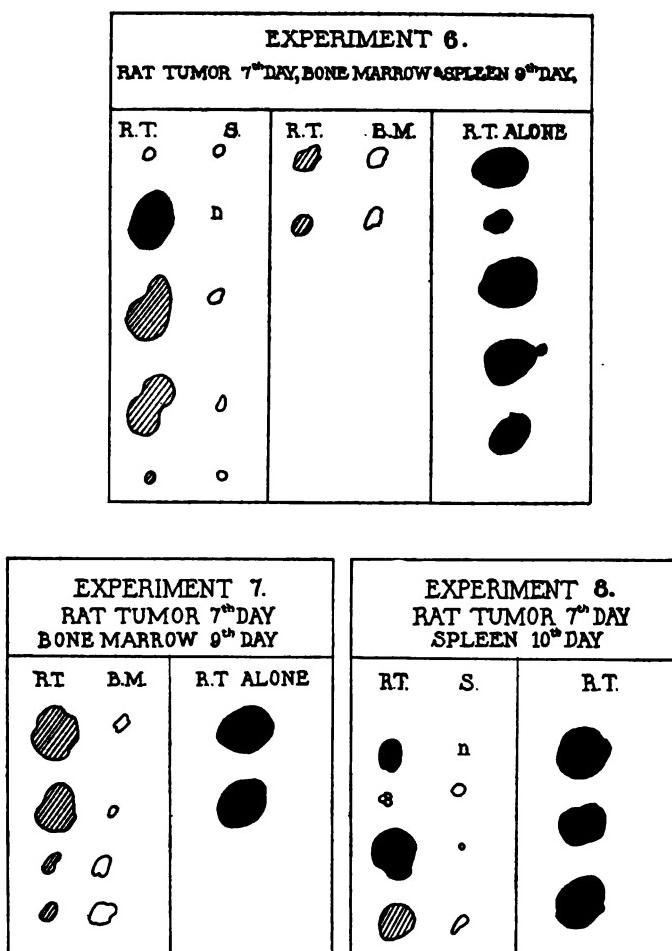
spleen graft well established, made up of the typical spleen cells. The rat tissue was found much degenerated, surrounded by collections of small round cells and largely replaced by connective tissue (figure 2). Later stages show the rat tissue to be dead, embedded in a mass of connective tissue with clumps of small round cells scattered throughout the surrounding tissue (figure 3). This condition offers a strong contrast to the picture shown by the controls of the rat tumor inoculated alone. Here the rat cells are practically devoid of stroma and the edges show no reactive tissue (figure 4). The final stages of rat tumor and chicken spleen showed the spleen graft to be in good condition, but with no evidence of the rat tissue remaining (figure 5).

The action of bone marrow resembled that of the spleen (figure 6), but was slower and less complete. The bone marrow grafts were composed for the most part of fat cells and collections of lymphoid cells.

#### THE EFFECT OF CHICKEN SPLEEN AND BONE MARROW ON ESTABLISHED GRAFTS OF FOREIGN TISSUES IN THE EMBRYO.

The fact that the tissues in the foregoing experiments were growing side by side and often intermingling presents a difficulty in the interpretation. The present series of experiments was planned to avoid the contact action and to give the spleen and bone marrow a more severe test. The kidney (figure 7) and other tissues mentioned above have no evident effect even in contact and they were therefore not used in this experiment.

Series of eggs were inoculated with rat sarcoma in the usual way on the seventh day of incubation. Two or more days later an opening was made on the opposite side of the eggs, and an adult chicken spleen or bone marrow graft was placed in the outer membrane. Some of the results are shown in outline in text-figure 2. On examination of these specimens eleven days later, the controls inoculated with rat tumor alone showed, almost without exception, large, well rounded, semitranslucent masses at the point of inoculation. In the embryos carrying a graft of adult spleen or bone marrow the tumors were flat, yellowish, and opaque. In some of these only a flake of tumor survived (figure 8). Microscopic ex-



TEXT-FIG. 2. This chart shows in silhouette the effect of adult chicken spleen and bone marrow on established and growing rat tumor in the embryo, when the adult tissues were inoculated at a distance. In the column with double rows of silhouettes the one on the left is the rat tumor (R.T.) and that on the right the bone marrow (B.M.) or spleen (S.) in the same embryo. The last column gives the controls of rat tumor alone. The day of incubation at which the inoculation was made is given in the caption. All tumors were removed at the eighteenth day of incubation. Black indicates that the tumors are composed of rat cells in active proliferation; the shaded outlines, that the rat tissue is much degenerated, with pronounced infiltration with round cells (figures 9 and 10). The unshaded outlines indicate that none of the rat cells survived. N indicates that graft did not take.

amination of the tumors showed massive collections of lymphocytes around the edges and in clumps associated with the blood vessels throughout the tumor (figures 9 and 10). There was a great increase in the connective tissue surrounding and replacing the rat tissue. The rat cells showed many degenerated forms and mitotic figures were rare. It would seem therefore that the adult spleen and bone marrow are capable of supplying a defensive mechanism to the chick embryo, even though the graft of spleen or bone marrow be some distance from the foreign tissue and introduced after the foreign tissue is established and actively growing.

#### DISCUSSION.

I shall make no attempt to discuss these findings in relation to the theories already brought forward to explain the failure of heteroplastic tumor grafts. The constant result obtained in a score of experiments or more shows conclusively that the adult spleen and bone marrow are capable of supplying a defensive mechanism to the chick embryo, which under ordinary conditions offers no resistance to the growth of foreign species tissue. Furthermore, the embryo bearing such grafts of spleen and bone marrow defends itself in the same way as the adult, if we may judge from the histological picture. The cells common to the graft of bone marrow and spleen, to the reaction around the foreign species graft in the adult, and to the embryo supplied with a defensive mechanism is the small lymphoid cell. It is therefore natural to suppose that this is an active agent in the defense.

Whether or not these lymphoid cells are the important factors in the failure of homoplastic grafts under certain conditions remains to be seen. Certainly a large preponderance of the evidence points in this direction. They are present often in large numbers around grafts of transplantable cancer in immune animals of the same species, regardless of the type of immunity. A recent communication of Apolant's<sup>11</sup> adds weight to this idea. He has shown that in splenectomized animals only a slight or very transient immunity can be developed to transplantable tumors. Oser and Pribram<sup>12</sup>

<sup>11</sup> Apolant, H., *Ztschr. f. Immunitätsforsch.*, *Orig.*, 1913, xvii, 219.

<sup>12</sup> Oser, E. G., and Pribram, E. E., *Ztschr. f. exper. Path. u. Therap.*, 1912, ii, 295.

have shown also that transplantable tumors grow more rapidly in splenectomized animals. The results reported by Baeslack<sup>18</sup> indicate that the number of small lymphocytes in the circulating blood of a tumor-bearing animal has a definite relation to the rate of growth of the tumor, falling rapidly in a susceptible animal and increasing steadily in an animal showing resistance.

#### SUMMARY.

It has been shown that the chick embryo offers suitable conditions for the growth of implanted tissues, whether these be embryonic or adult, of the same species or a foreign one. The chick at about the time of hatching develops a defensive mechanism against the tissue of foreign species. This resistance can be supplied to the embryo in the early stages if grafts of adult spleen or bone marrow are implanted. Under these conditions the embryo exhibits the same resistance to foreign tissue as does the adult, and presents the same histological manifestations about the graft. Furthermore, the same tissues, spleen and bone marrow, when grafted into an embryo with an established and growing rat tumor, bring about a retrogression and absorption of the foreign tissue. Other adult tissues do not supply this power to the embryo.

#### EXPLANATION OF PLATES.

##### PLATE 56.

FIG. 1. The edge of a tumor resulting from a ten days' growth of a Jensen rat sarcoma and adult chicken kidney inoculated into the outer membrane of a seven day embryo. The kidney tubules are seen scattered around the edge of the tumor mass, which is made up of the rapidly grown rat cells.

FIG. 2. A section of a tumor resulting from a simultaneous inoculation of adult chicken spleen and a rat sarcoma, after five days' growth in the outer membrane of a chick embryo. A = spleen graft. B = the sarcoma cells surrounded and largely replaced by small round cells and connective tissue.

##### PLATE 57.

FIG. 3. The remains of the rat sarcoma cells eight days after inoculation into an embryo which at the same time received a graft of adult chicken spleen. The cell structure of the rat tissue is entirely lost and the whole is embedded in a thick connective tissue mass. Small round cells are seen scattered through the section. Compare with figure 4.

<sup>18</sup> Baeslack, F. W., *Ztschr. f. Immunitätsforsch., Orig.*, 1914, xx, 421.

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FIG. 4. A section of a tumor resulting from an inoculation of the rat sarcoma alone, after eight days' growth in the chick embryo. Several mitotic figures are seen.

PLATE 58.

FIG. 5. The resulting tumor from a simultaneous inoculation of a seven day embryo with a rat sarcoma and a graft of adult chicken spleen, after eleven days. The spleen graft is seen on the left and the location of the sarcoma graft is on the right. There are no evidences of the rat cells remaining.

FIG. 6. This section shows the effect of a chicken bone marrow graft on a rat sarcoma after six days in the embryo. The rat cells (A) are embedded in a mass of small round cells (B).

PLATE 59.

FIG. 7. A drawing, somewhat enlarged, showing a tumor in the outer membrane of an eighteen day old embryo, resulting from a simultaneous inoculation eleven days previously of grafts of rat sarcoma and adult chicken kidney. The kidney is shown as the bluish nodule in the concavity of the tumor. The controls of rat tumor alone ranged about the same size.

FIG. 8. A drawing, somewhat enlarged, showing the effect of adult chicken spleen on an established graft of rat tumor in a chick embryo. The lower figure is the control of rat tumor alone after eleven days of growth in the outer membrane of chick embryo. The upper figure is the outer membrane of an embryo inoculated at the same time as the above with rat tumor (yellowish area), but two days later a graft of adult chicken spleen was added (pink nodule).

PLATE 60.

FIG. 9. Section of rat tumor in a chick embryo which had at some distance away a graft of adult bone marrow. A = round cell infiltration. B = degenerated rat cells. Compare with figure 4.

FIG. 10. Section of rat tumor in a chick embryo which had at some distance away a graft of adult chicken spleen. A = dense round cell infiltration at edge. B = degenerated rat cells lying in a mass of connective tissue. Compare with figure 4.

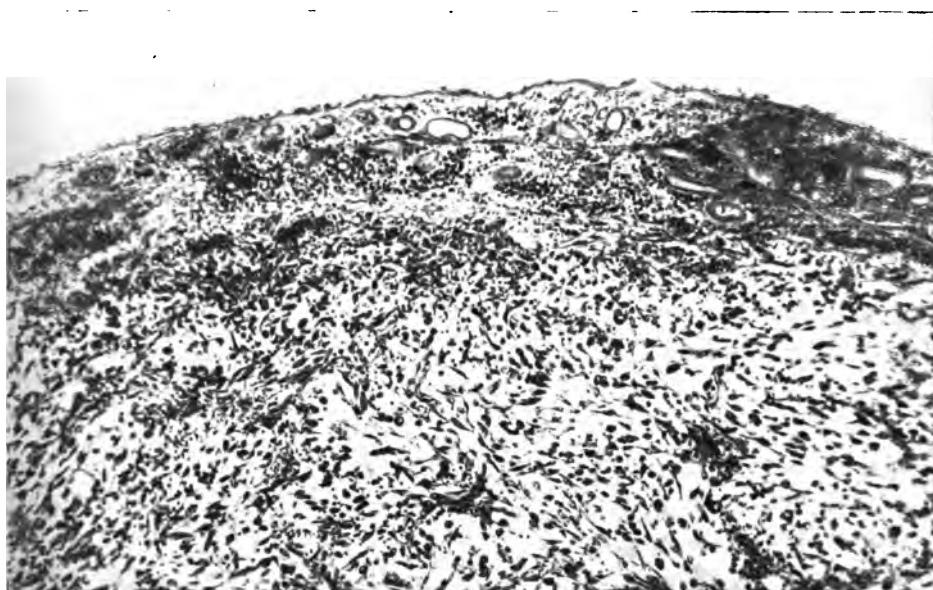


FIG. 1.

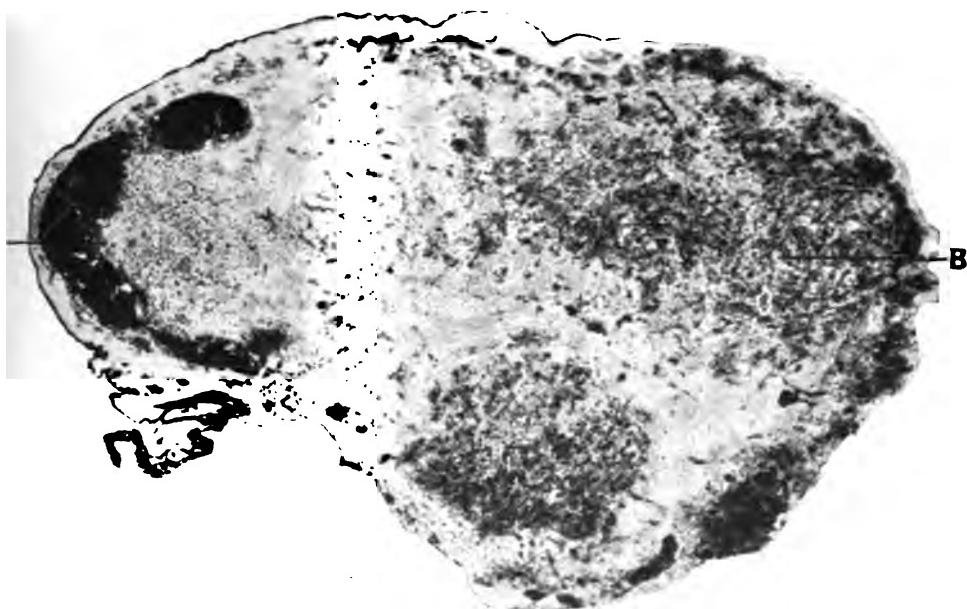


FIG. 2.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)



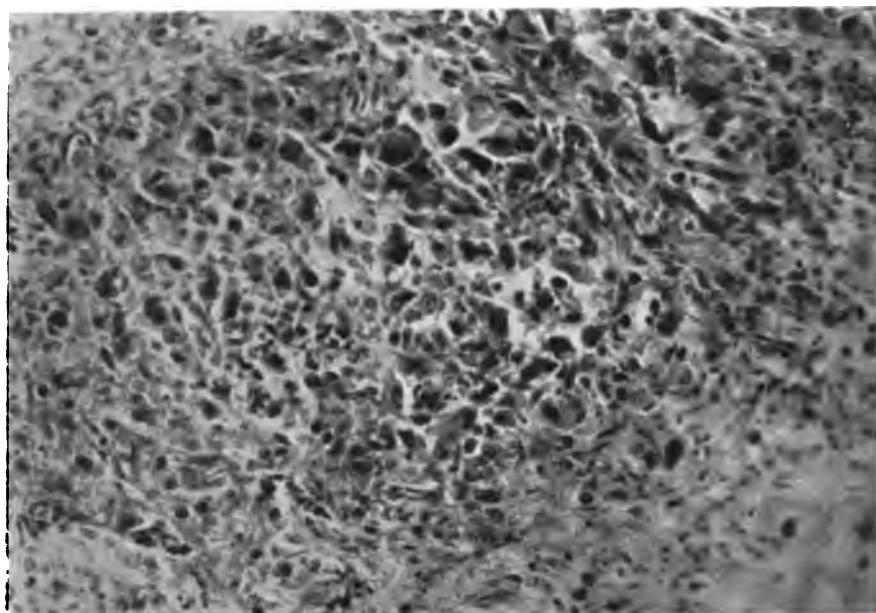


FIG. 3.

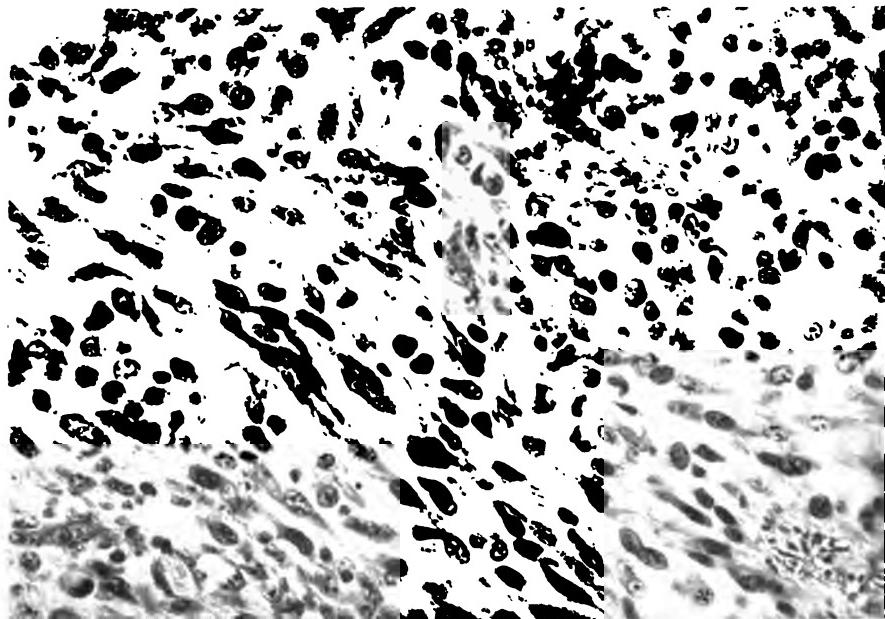


FIG. 4.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)



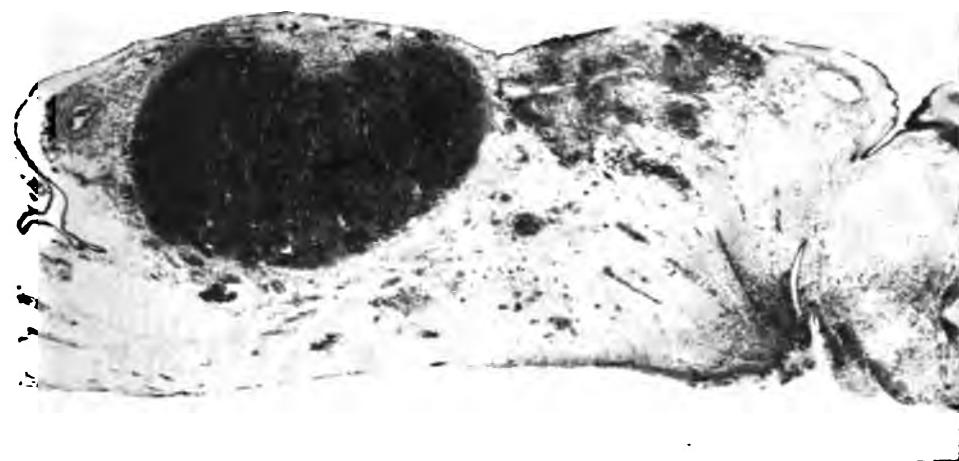


FIG. 5.

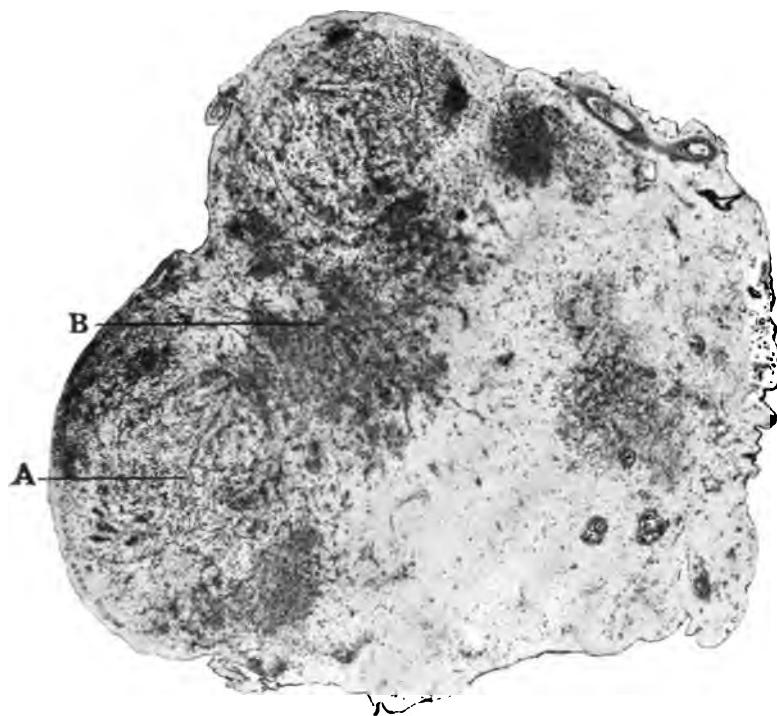


FIG. 6.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

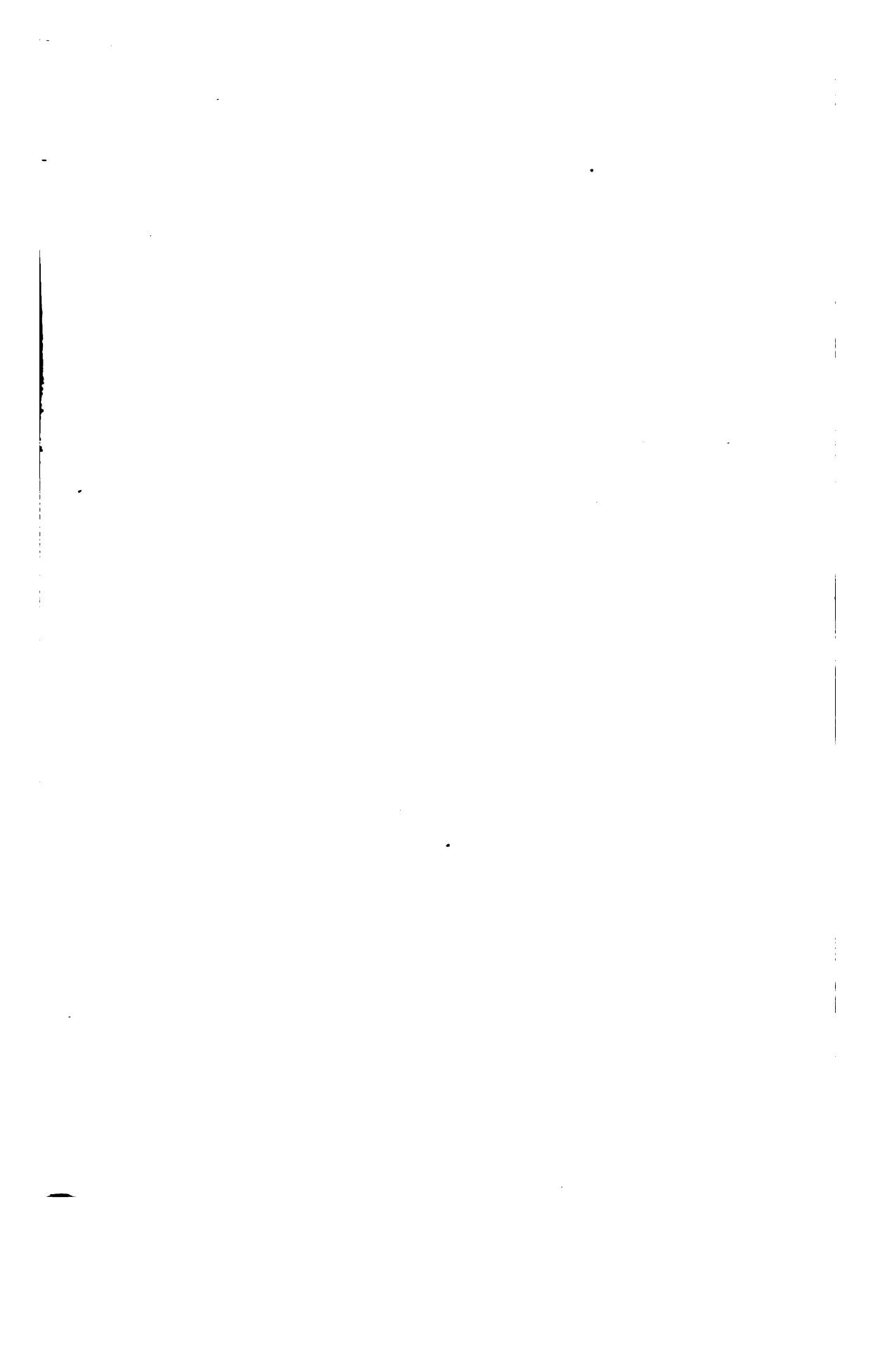




FIG. 7

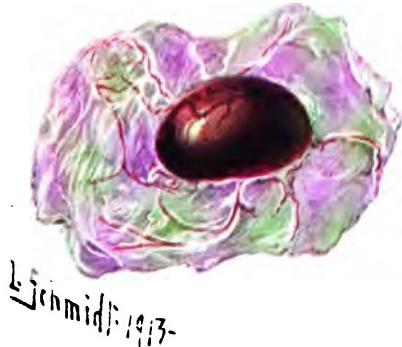
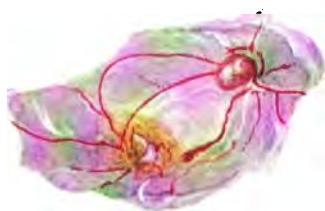


FIG. 8.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)



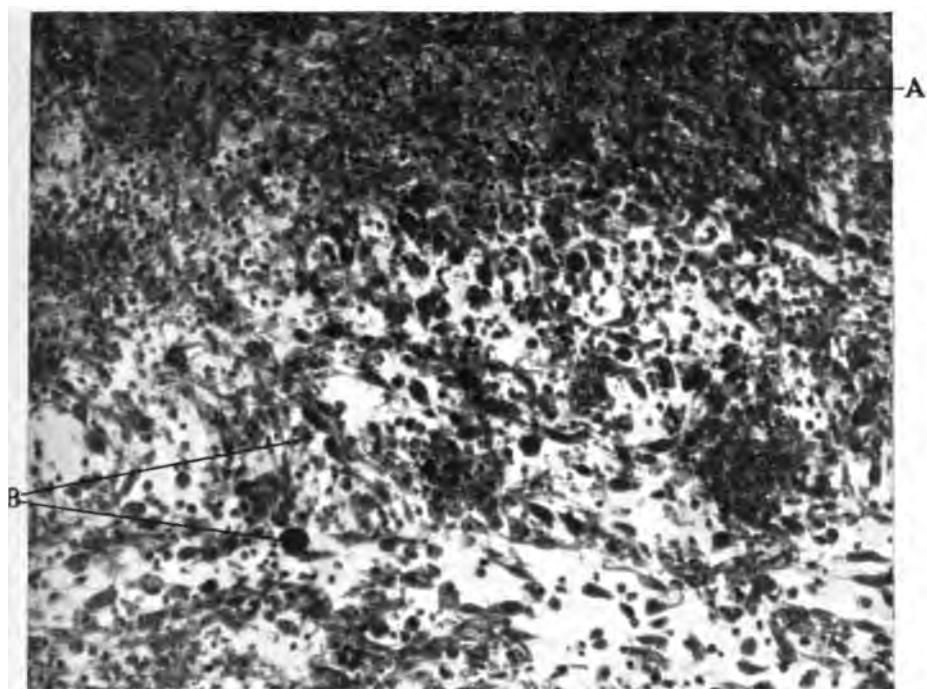


FIG. 9.

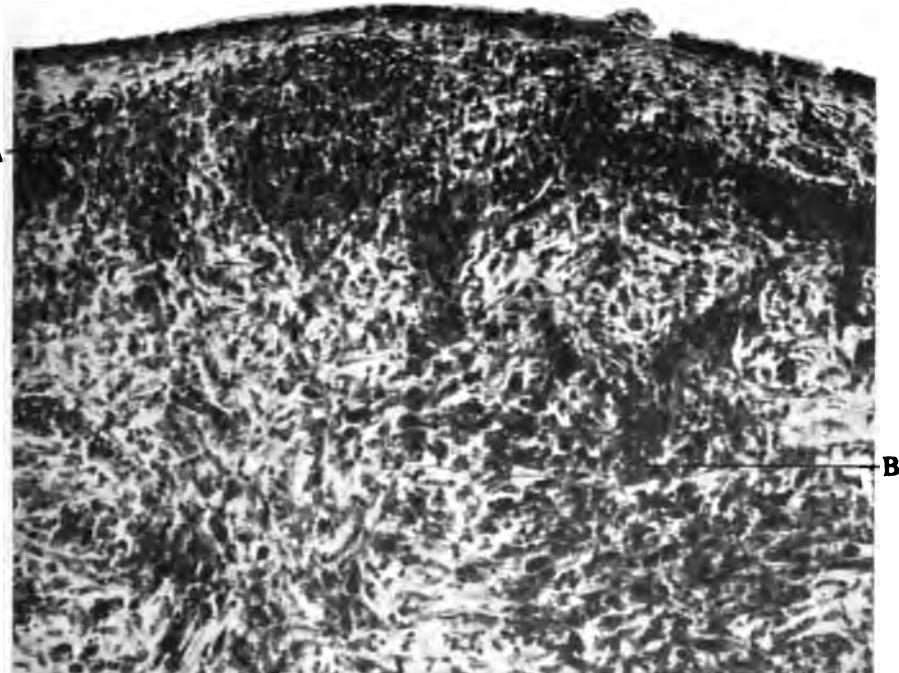


FIG. 10.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)



# FREE MALARIAL PARASITES AND THE EFFECT OF THE MIGRATION OF THE PARASITES OF TERTIAN MALARIAL INFECTIONS.\*

By MARY R. LAWSON, M.D.

(*From the Laboratory of Dr. Mary R. Lawson, New London.*)

PLATES 61 TO 66.

I have shown in a previous paper<sup>1</sup> that the malarial parasite is extracellular throughout its life cycle; that in the course of its evolution, it may destroy a number of red corpuscles, migrating from one to another; and that in the intervals between its abandonment of the degenerated remnants of the red corpuscles (corpuscular skeletons) and its subsequent attachment to the surface of fresh red corpuscles, the parasite is, for very brief periods of time, free in the blood serum. The destruction of several red corpuscles by each parasite would account for the rapidity and severity of the anemia occurring in malarial infections.<sup>2</sup>

The free tertian parasites pictured in this article (with the exception of figures 96 to 99, 109 to 112, 114 to 116, 121, 122, and 124) came from two cover-slip smears; one of the specimens is from a series of smears taken at intervals of approximately one half minute, the other from a series of smears taken at intervals of one half hour. No quinine had been given.

In order to make clear the relation of the free parasites to migration, I shall describe briefly the process of migration.

## *Various Stages in the Migration of the Tertian Malarial Parasite.*

—(1) Pigmented parasites on decolorized red corpuscles or on corpuscular skeletons (granular degenerated remnants of red corpus-

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<sup>1</sup> Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420.

<sup>2</sup> Marchiafava and Bignami (1) write: "In no other infection is anaemia produced with the same rapidity and to the same extent as in malaria."

cles); (2) pigmented parasites in the act of abandoning the decolorized or granular degenerated red corpuscles; (3) pigmented parasites, free in the blood serum; (4) pigmented parasites in the same stages of development as the parasites described under stages 1, 2, and 3, but attached to red corpuscles, the hemoglobin of which appears to be as yet unaltered.

*The Rapidity with Which Migration May Take Place.*—I believe the process of migration to be very rapid. The young unpigmented parasites usually appear almost immediately attached to red corpuscles. Thayer (2), in writing of these fresh segments, states: "They appear in the red corpuscles simultaneously with, or shortly after the appearance of the sporulating bodies." I believe that the free pigmented parasites in migration also attach themselves almost immediately to fresh red corpuscles. This belief is supported by the following facts: (a) all the above stages in migration may be present in one smear; and (b) only two successive smears, from the series of 200 smears taken at one half minute intervals, showed what I interpret as stages in migration.

*Parasites Free in the Blood Serum.*—At times there may be seen free in the blood serum segments resulting from a recent segmentation, and pigmented parasites in several stages of development and in various phases: (a) presegmenting (figures 110 and 111); (b) segmenting (figure 109); (c) individual segments (figures 36 and 96); (d) ring-form (figures 1 to 14, 48, 57, and 67); (e) ameboid (figures 62 to 64, 69, and 70); (f) compact (figures 16 to 35, 38 to 40, 47, 49, 51, 114 to 120, and 126); (g) with protoplasmic pseudopodia (figures 37, 41 to 46, 50, 52 to 56, 58 to 61, 65, 66, 68, 71 to 94, 95 to 102, 104 to 108, 112, 113, 122, 123, 125, 127, 128); (h) with flagella (figure 113).

*Presegmenting Parasites.*—This type of parasite is so often found free, that I believe the parasite must always migrate in this stage of development.

*Segmenting Parasites.*—In these instances the smear must have been taken just as the corpuscle was destroyed, and before the individual segments had time to take up their separate existence.

*Individual Segments.*—It is not uncommon to see the parasites free in this stage of development; but it is extremely rare to find one of them with a pseudopodium (figure 96).

*Ring-Form Parasites.*—These parasites, on destroying the red corpuscles to which they were attached, have been freed in the form which they had assumed while surrounding a corpuscular mound (figure 15).

*Ameboid Parasites.*—These parasites have either been freed in this form after having destroyed the corpuscle to which they were attached, or have assumed it in preparation for a new attachment. Parasites in this phase are often difficult to distinguish from parasites with protoplasmic pseudopodia.

*Compact Parasites, or Parasites Which Have Contracted Their Protoplasm into a Discoid or Spheroidal Form.*—These are the types of free parasites most commonly seen both in fresh blood preparations and in stained specimens, and the compact form is, I believe, the normal resting form of the parasite when living, and that usually observed when the parasite is dead. It is in this form that the parasite is generally found in the blood of cinchonized patients, and in smears from the cadaver.

In referring to this form of the living parasite, Laveran (3) states that spherical bodies are at times free in the blood serum, and he pictures them in several stages of development. Manna-berg (4) writes: "The perfectly globular form is first assumed by the parasite when it has escaped out of the red blood corpuscle." And Marchiafava and Bignami (5), in referring to these free compact parasites, state: "We would call attention to the presence of pigmented spherules of varying sizes, which are almost always found free in the blood of a tertian patient. They are of importance in diagnosis, because sometimes they exist alone [migration?], the most careful examination failing to reveal any endoglobular parasitic bodies. These spherules are composed of a hyaline substance with small granules of freely moving pigment, and possess so characteristic an appearance to the expert eye that they quite suffice to a diagnosis of malarial tertian. For the greater part they are merely parasites which have escaped from the red corpuscle."

I have not made an exhaustive study of specimens of blood taken after the patient had been given quinine; but such smears as I have studied showed an almost complete absence of parasites. I have seen free compact parasites after the use of quinine; but I do not

believe that these parasites were in migration, for they showed no such uniformity in their stages of development in any one smear as healthy parasites in migration ordinarily do, nor did they take the stain as vividly as did the healthier parasites. I believe that it was the action of the quinine that caused these parasites to abandon the red corpuscles and assume the compact form. Marchiafava and Bignami (6) state that quinine may cause certain parasites to abandon the red corpuscles, and that (7) ring-form bodies disappear from malarial blood which has been strongly cinchonized. Romanowsky (8) describes "quinine forms" in which the clear zone of the nucleus was wanting [the corpuscular area], this structure fading insensibly into the body of the parasite"; and Golgi (9) claims that after quinine, the body of the tertian parasite becomes round and motionless. I believe that in these instances the parasites were either sick or dead.

In smears from the cadaver I have seen free parasites only in the compact form. Indeed Marchiafava and Bignami (10) go so far as to assert that "in the cadaver we do not see annular bodies; but only, as a rule, immotile discoid or spherical micrococciform bodies." In these instances I believe that the compact form is the result of the death of the parasites.

*Parasites with Protoplasmic Pseudopodia.*—These are parasites in varying stages of development, with attaching pseudopodia which are derived from the cytoplasm of the parasite. These pseudopodia may vary both in length and in number, and may or may not contain pigment granules. I believe that the pseudopodia are used by the parasites to secure their prey, as well as for the purpose of attachment to the red corpuscle. These attaching processes must be sharply distinguished from flagella, which arise from the chromatin substance of the parasites. Free parasites with pseudopodia are only rarely seen in stained smears from the circulating blood. They are most commonly thrown out when the parasite is free from the corpuscle; but very rarely one or more of the filaments may be seen going off beyond the periphery of the degenerated red corpuscle to which the parasite is still attached (figures 103 and 125). Dock (11) has also described and pictured the pseudopodium of a parasite going off beyond the periphery of the red corpuscle to which the parasite was attached.

Celli and Guarnieri, and later also Plehn (12), believed that they saw spores in the blood serum which "swarmed by the means of flagella" (figure 96 shows a spore with a flagellum), and Thayer (13) has observed that the "small hyaline bodies may sometimes be followed for some little distance from their original segmenting form. Under these circumstances they may show a slight dancing to-and-fro movement which suggests the possible existence of flagella."

*Parasites with Flagella.*—These parasites are microgametocytes in the sexual phase, the flagella arising from the chromatin substance of the parasite. In stained specimens, as a rule, the sexual flagella have a more curled appearance than do the protoplasmic attaching processes. In my experience it is rare to find pigment in connection with flagella. Flagellation may occur either when the parasite is free from the red corpuscle, or when it is still attached to it. Preflagellating parasites are occasionally found free, and may be seen with or without attaching filaments (figure 112, with attaching pseudopodia). This form of parasite is usually smaller than the segmenting bodies in the same infection, is deeply stained, and contains more chromatin in proportion to its protoplasm.

Sexual flagellating parasites are only occasionally found in stained smears from the circulating blood, but now and then a few examples may be seen. Rarely one is fortunate enough to find the complete sexual cycle.<sup>3</sup> I have found actively sexual parasites in all types of malaria, but only in cases showing very heavy infections. It seems to me that the term flagellata is a misnomer when applied to parasites in the sexual phase; but as it is the term which is generally accepted as applying to parasites in this phase, I continue to use it.

Sexual flagellating parasites should not be confused with parasites extruding protoplasmic pseudopodia. I believe that many of the so called flagellating bodies seen in fresh blood preparations are not parasites in the sexual phase, but parasites which, on destroying the corpuscle to which they were attached and becoming free, proceed to throw out protoplasmic processes for the purpose of capturing and reattaching themselves to other red corpuscles. It has often

<sup>3</sup> Rowley-Lawson, M., *Jour. Exper. Med.*, 1911, xiii, 263.

been noted that the red corpuscle is practically destroyed before the parasite abandons it and then proceeds to flagellate. Why should the corpuscle be destroyed before the parasite flagellates, if this flagellation is always a sexual phase? Does the parasite time its destruction of a red corpuscle to correspond with, or nearly with, the developmental stage of a sexual flagellation? I have stained some of these so called flagellating bodies and found the filaments composed of protoplasm only, and several observers have stated that the flagella observed by them appear to be protoplasmic in nature and that the nucleus does not take part in the process of flagellation. Antolisei (14) believes flagellation to be a degenerative process; the flagella he describes as sarcodic prolongations of the protoplasm. Grassi and Feletti (15) state that the nucleus does not take part in the process of flagellation, neither dividing nor entering into the flagella. Marchiafava and Bignami (16) found that "there are some flagellata in which the filaments do not contain any chromatin; but are composed of protoplasm alone. And others in which there may be one or two filaments provided with chromatin, the others being formed of protoplasm." Figure 113 shows a free flagellating parasite, with one filament composed of chromatin (a); the other filaments (o) are formed from the cytoplasm of the parasite, and pigment granules may be seen in connection with them.

Sakharoff (17) describes what I believe to be genuine sexual flagellating parasites. "The process of the formation of the flagellate bodies, consists in a perversion of the karyokinetic nuclear division, in a breaking up of the nucleus into the chromatin filaments, and in the escape of these from the parasite; these filaments which are in lively motion represent the flagella." Sakharoff believed this process of flagellation to be a degenerative one.

Malarial parasites in various stages of development have been noted and pictured free in the blood serum by so many observers, that it is surprising that no rational explanation has been offered to account for them. Laveran (18) constantly refers to them, stating that they may "often be found in the blood in a free state, and that at all periods of their development." Mannaberg (19) writes: "The spores which have become free . . . are often to be demon-

strated in enormous numbers. . . . The amoeboid bodies too, which are developed further than their spore-forming stage, are not infrequently to be found free in the liquor sanguinis, fully retaining their structure." Thayer and Hewetson (20) state: "Occasional pigmented bodies may be found outside the red corpuscles, free in the plasma. Some of these represent full-grown bodies which have destroyed the surrounding corpuscle, while in other instances, the half-grown forms may actually leave their host." Ewing (21), in his monograph on malarial parasites, states that Celli and Guarneri and Ziemann have depicted free parasites and that he himself has seen them in fresh blood preparations; but he believes that they are extremely rare in circulating blood. He writes: "In preparations of fresh blood, parasites so frequently pass from the cell into the plasma that it may be doubted if any accurate estimation of the number of extra-cellular bodies in the circulating blood can be obtained by this method of examination"; and Ruge (22) has pictured free parasites in several stages of development.

Bass and Johns (23) claim that the malarial parasites "cannot live for even a few minutes free in the serum." Of course this statement must apply only to parasites in artificial media or under very abnormal conditions, for all observers know that parasites are seen to remain in motion and apparently alive for many minutes free in the serum. Laveran (24) has noted that they may remain alive for half or three quarters of an hour. Marchiafava and Big-nami (25) have noted that in flagellated bodies the movements of the pigment granules within the pigmented body "may continue for hours." Thayer and Hewetson (26) note that motions may be observed in flagellate bodies sometimes for as long as half an hour, the flagellating bodies being free; for they (27) state, "We have never seen the appearance of flagella in bodies still contained in the red blood corpuscles." Schaudinn (28) claimed to have watched under the microscope the "entrance into a red corpuscle" of a young parasite resulting from a segmentation, and I have frequently seen free parasites remain alive for over half an hour. The segments from a sporulating body as well as the parasites which are injected by the infecting mosquito must also be free in the blood serum for a certain length of time.

If all malarial parasites are, as I believe, attached to the external surface of the red corpuscles,<sup>4</sup> they must constantly be exposed to the action of the patient's serum. Stained specimens seem to show that the free parasites in migration do not remain long free, but attach themselves almost immediately to fresh red corpuscles; and if we stop to consider how short the life of the individual parasite is, even the brief periods of time when it is free in the blood serum may be comparatively long. Hence one would expect, under normal conditions for the parasite, what to us seems an immediate attachment.

*The Effect of Migration, the Progressive Loss of Red Blood Corpuscles in the Intervals between Paroxysms in Tertian Malarial Infections.*—Several years ago, while trying to estimate the degree of anemia in certain cases of tertian malaria showing very heavy infections, the late Dr. J. H. Donoghue of Boston and I took red counts at certain intervals, from one segmentation period to seven to eight hours after the following segmentation period, before giving quinine. The segmentation period was determined from stained specimens and not estimated from the time of the chill. We found that the anemia progressed between the paroxysms, that the greatest fall in the red corpuscle count occurred, not, as we had expected, soon after the segmentation of the parasites with the resulting destruction of red corpuscles to which they had been attached, but from six to seven hours later. Unfortunately we did not control each intermediate count of red corpuscles with a stained specimen. Later, in going over the stained specimens from these cases, I found in some of the smears certain free parasites, all in a few definite uniform stages of development. I did not at first connect these free parasites with the progress of the anemia between the paroxysms; but later I interpreted them as migratory parasites capable of destroying more than one red corpuscle. In these cases blood regeneration began as soon as quinine was given.

The maximum fall in the red count several hours after each segmentation period is due, I believe, not only to the loss of red corpuscles resulting from the segmentation of the parasites, but also to the subsequent and further destruction of corpuscles by the new

<sup>4</sup> Rowley-Lawson, M., *Jour. Exper. Med.*, 1913, xvii, 324.

group of parasites. Kelsch (29) observed the progress of anemia between the paroxysms, and stated (30) that in a robust individual, in the course of one day, the number of red corpuscles may go from normal to 1,000,000 per cubic millimeter. Dionisi (31) observed a continuous loss of red corpuscles during the afebrile interval, and Ewing (32) states that the anemia may progress during afebrile periods. I believe that quinine must be kept up for longer periods than is usual in order to insure a complete elimination of the parasites, otherwise we may be confronted with relapses. Although the malarial parasites may show a variable resistance to quinine, I do not believe that there is any stage in their development or phase in their existence when they are absolutely impervious to persistent and long continued treatment.

#### SUMMARY.

1. The malarial parasite is extracellular throughout its life cycle and migrates from red corpuscle to red corpuscle destroying each before it abandons it; in the brief intervals between, the parasite is free in the blood serum; it does not remain long free, but almost immediately attaches itself to another red corpuscle by means of delicate pseudopodia.
2. The compact form of the tertian parasite is the type of free parasite most often observed; in this form the parasite may be seen not only in migration, but after quinine and in the cadaver. I believe the compact form to be the normal resting form of the parasite, all other forms being assumed in order to secure attachment and to obtain food.
3. Care must be taken not to confound free parasites having protoplasmic pseudopodia ready for attachment with the sexual flagellating parasites, whose flagella are composed of chromatin.
4. The malarial parasite can live for some time free in the blood serum, though under normal conditions there is no reason why it should remain free for any length of time, and there are certain periods in the life of the parasite when it must be admitted that it is free from the corpuscle and survives. If the parasite is, as I believe, attached to the external surface of the red corpuscle, it is constantly exposed to the action of the patients' serum.

5. The destruction of more than one red corpuscle by each parasite would readily account for the severe and early anemia occurring in malarial infections.
6. Long continued treatment with quinine will eventually cause the death of all malarial parasites.

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## EXPLANATION OF PLATES.

## PLATE 61.

## FREE TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,725$ .

FIGS. 1 to 14. Pigmented ring-form parasites freed in the form which they had assumed when encircling corpuscular mounds. In figure 4 a very delicate pseudopodium may be seen at x. In figure 13 the periphery of the adjacent red corpuscle shows through the opening of the ring.

FIG. 15. The ring-form is seen encircling the corpuscular mound of a red corpuscle.

FIGS. 16 to 35. Various pigmented compact parasites with contracted protoplasm. Figure 20 illustrates what I believe to be a microgametocyte.

## PLATE 62.

## FREE TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,725$ .

FIG. 36. Two segments resulting from a recent sporulating body.

FIG. 37. A young pigmented parasite with an attaching pseudopodium.

FIGS. 38 and 39. Two young pigmented parasites with contracted protoplasm.

FIG. 40. A young unpigmented parasite which has, I believe, been freed prematurely from a red corpuscle, probably by a parasite of more advanced growth destroying the corpuscle.

FIGS. 41 to 45. Young pigmented parasites with protoplasmic pseudopodia out for attachment to fresh red corpuscles. In figure 44 the parasite appears to be already attaching itself to the red corpuscle.

FIGS. 46 to 70. Pigmented parasites, ring-formed, ameboid, compact, and with protoplasmic pseudopodia.

## PLATE 63.

## FREE TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,765$ .

FIGS. 71 to 94. Pigmented parasites with pseudopodia arising from the cytoplasm of the parasites.

## PLATE 64.

## FREE TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,725$ .

FIGS. 95 to 108. Parasites with pseudopodia arising from the cytoplasm of the parasites. Pigment granules may be seen in connection with many of the pseudopodia.

FIG. 96. A young segment with a pseudopodium. It is rare to find segments with pseudopodia in stained specimens.

FIG. 98. The pseudopodium is seen to be perfect in form and its length is twice the diameter of a red corpuscle.

FIG. 103. The pseudopodium of the parasite is seen extending beyond the periphery of the dehemoglobinized red corpuscle to which the parasite is still attached.

#### PLATE 65.

##### FREE TERTIAN MALARIAL PARASITES.

Magnification,  $\times 1,725$ .

FIG. 109. A segmented parasite free from a red corpuscle.

FIGS. 110 and 111. Presegmenting parasites free from red corpuscles.

FIG. 112. A preflagellating parasite (microgametocyte) free from the red corpuscle, with protoplasmic pseudopodia at x. Note that the parasite has been freed in the form which it had assumed when surrounding a corporeal mound. Compare this parasite with the presegmenting parasites and note that it contains more chromatin in proportion to its cytoplasm than do the presegmenting bodies.

FIG. 113. A sexual flagellating parasite (microgametocyte) with the flagella composed of chromatin at a, and pseudopodia from the cytoplasm at o. Granules of pigment may be seen in connection with the pseudopodia.

FIG. 114. This shows what I interpret to be a microgametocyte free from the red corpuscle. Careful examination will show what appear to be pseudopodia just starting out at the periphery of the parasite on the left.

FIGS. 115 to 120. Large free pigmented parasites. Note that the chromatin in figures 117 to 120 is very palely stained. I believe these parasites to be unengaged adult macrogametes.

FIG. 121. An adult pigmented parasite almost freed from a red corpuscle showing advanced granular degeneration.

FIG. 122. An adult pigmented and well stained parasite which was probably about to abandon the degenerated remnant of the red corpuscle to which it was attached before the spreading of the smear. The process of smearing the blood probably separated the parasite from the remnant.

FIG. 123. An adult pigmented parasite similar to those shown in figures 117 to 120, but with a pseudopodium, suggesting that the parasite is preparing for another attachment.

FIG. 124. Two parasites, an adult pigmented and a young unpigmented parasite, still attached to a red corpuscle showing advanced granular degeneration. I believe that the adult parasite is preparing to migrate and in doing so it will also free the young parasite, probably prematurely.

FIG. 125. A pigmented parasite preparing to migrate from a degenerated red corpuscle. Note the pseudopodium extending beyond the periphery of the red corpuscle.

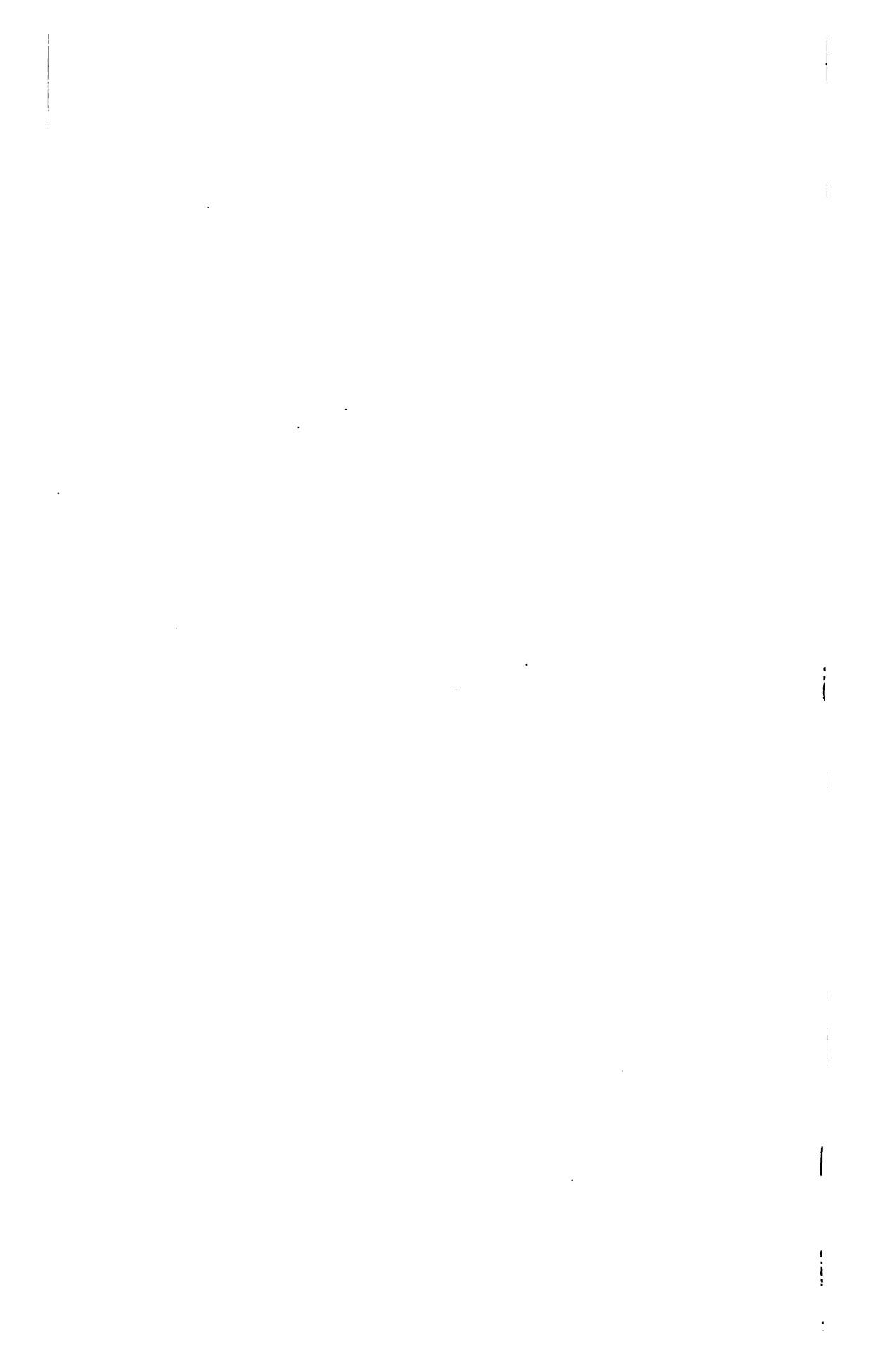
FIG. 126. A young parasite which appears to be only just freed from the remnant of a degenerated red corpuscle, shown just below the parasite.

FIG. 127. A free parasite with protoplasmic pseudopodia.

FIG. 128. A free pigmented parasite with protoplasmic pseudopodia.



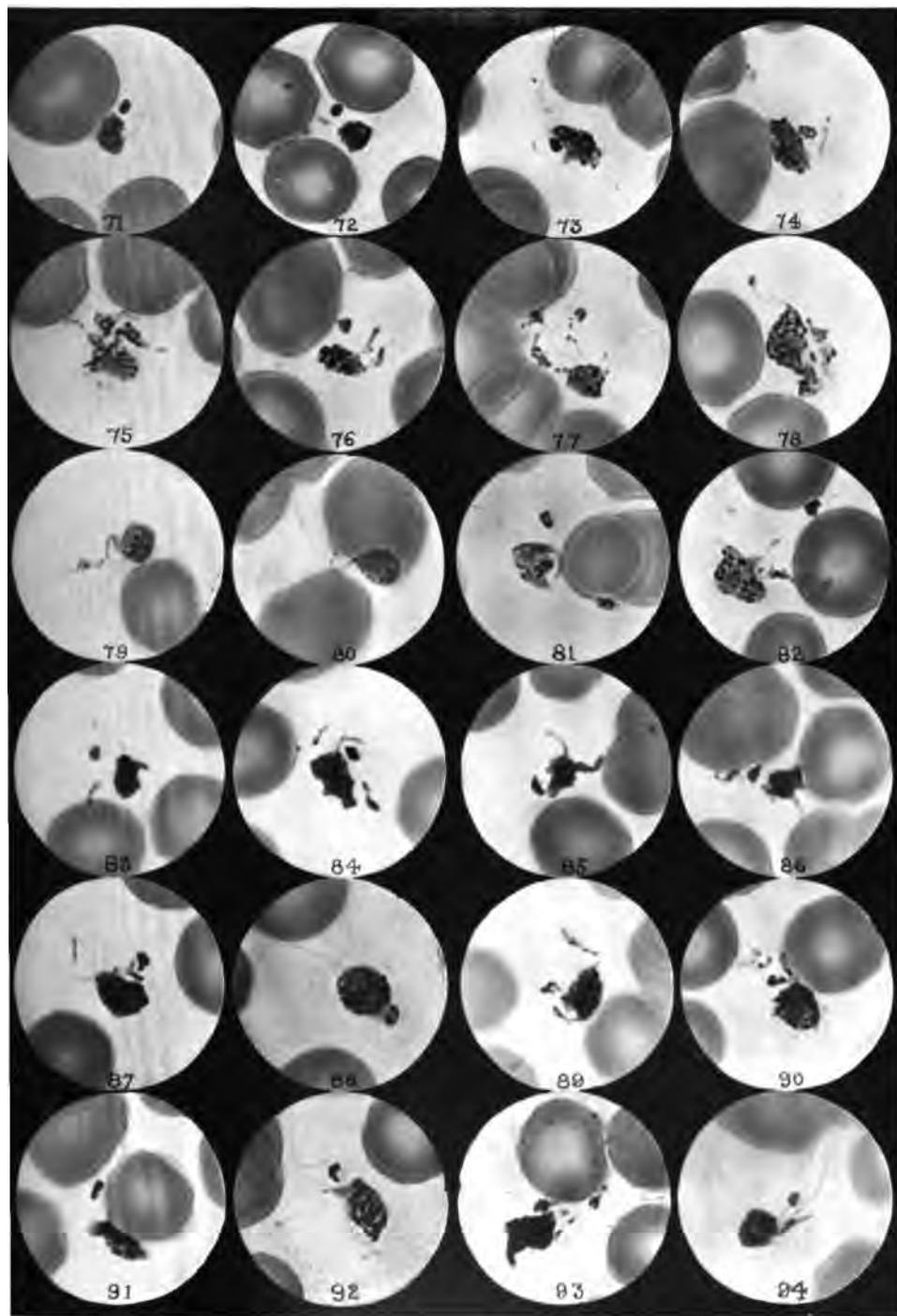
(Lawson: Free Malarial Parasites.)



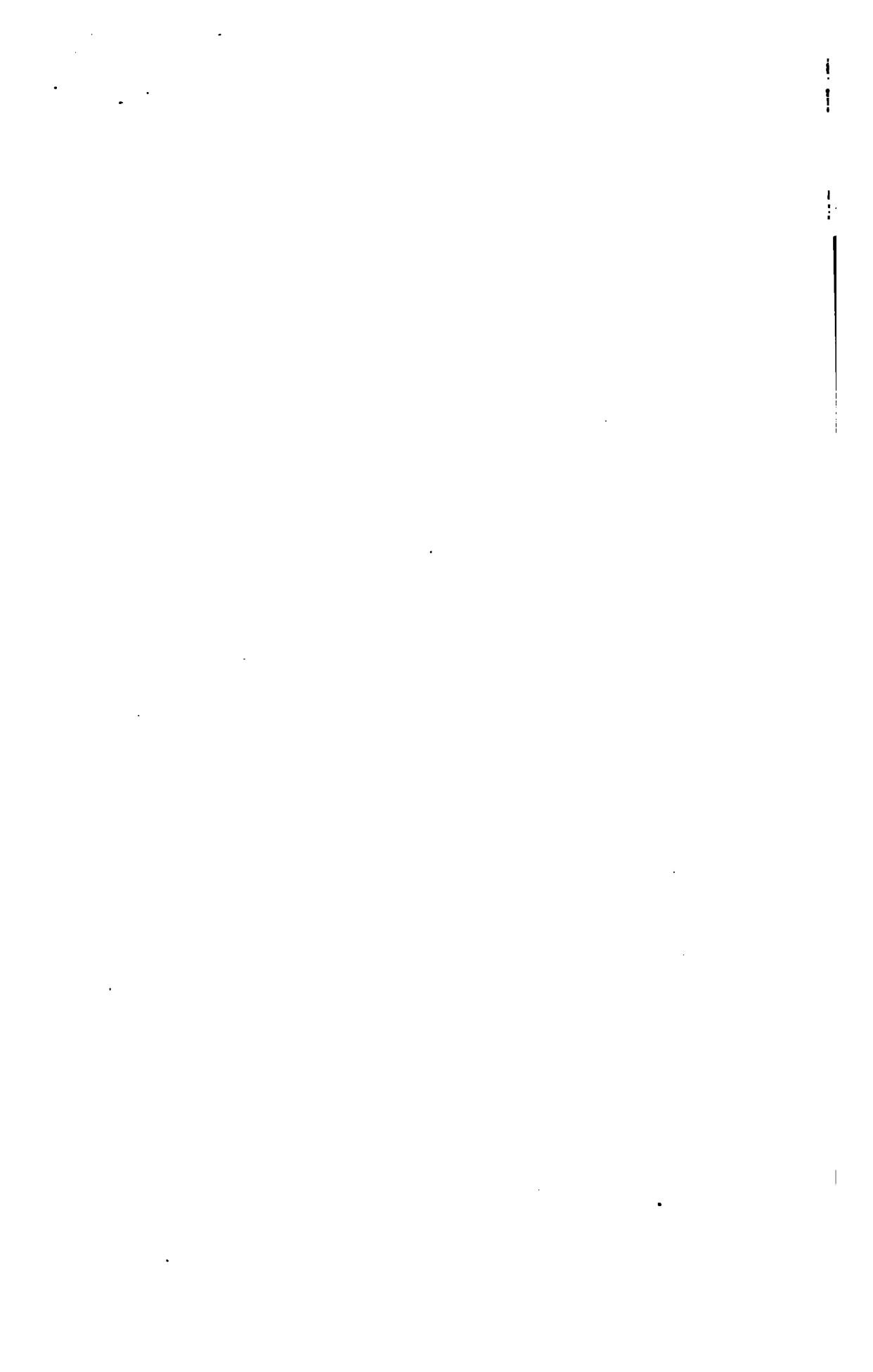


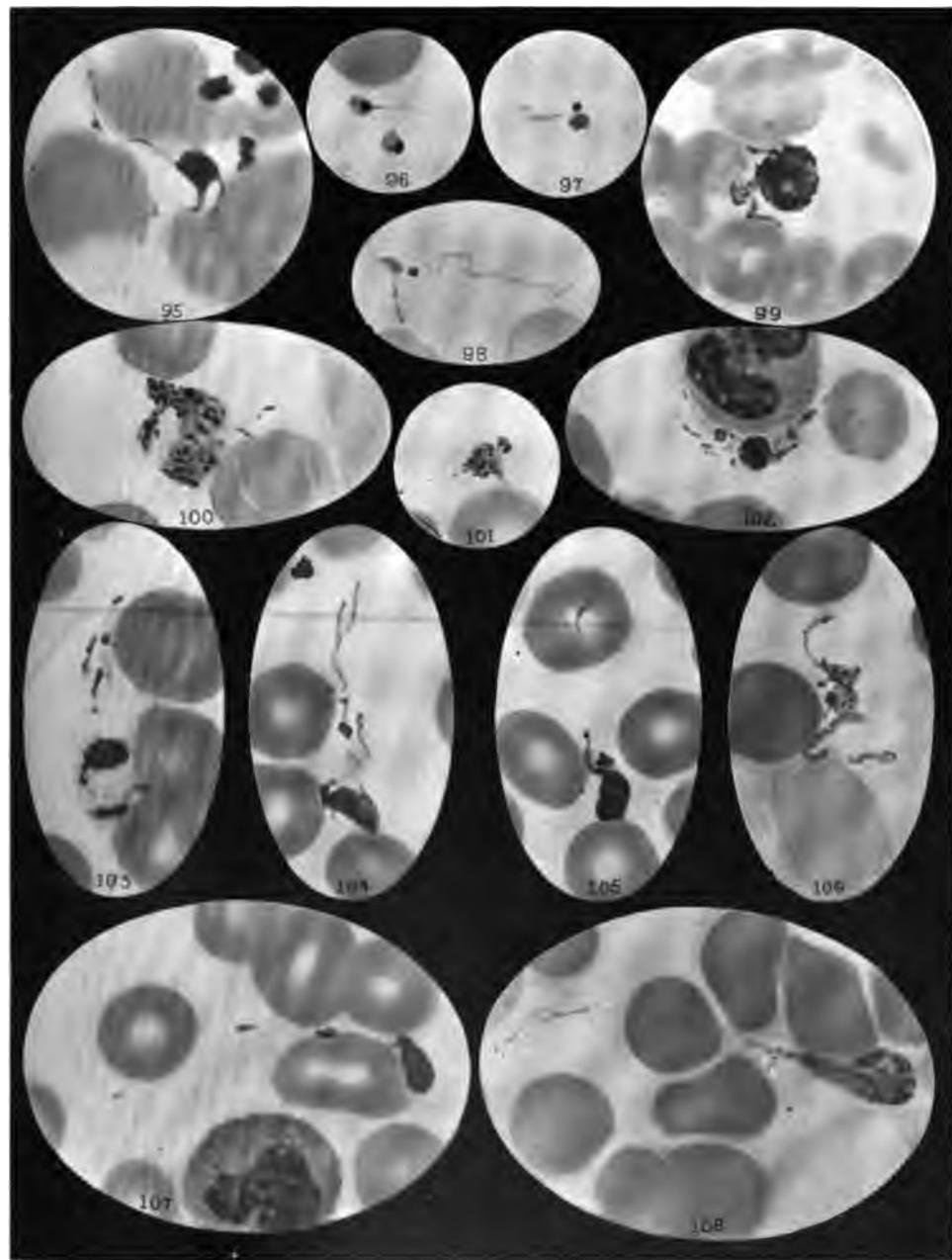
(Lawson: Free Malarial Parasites.)



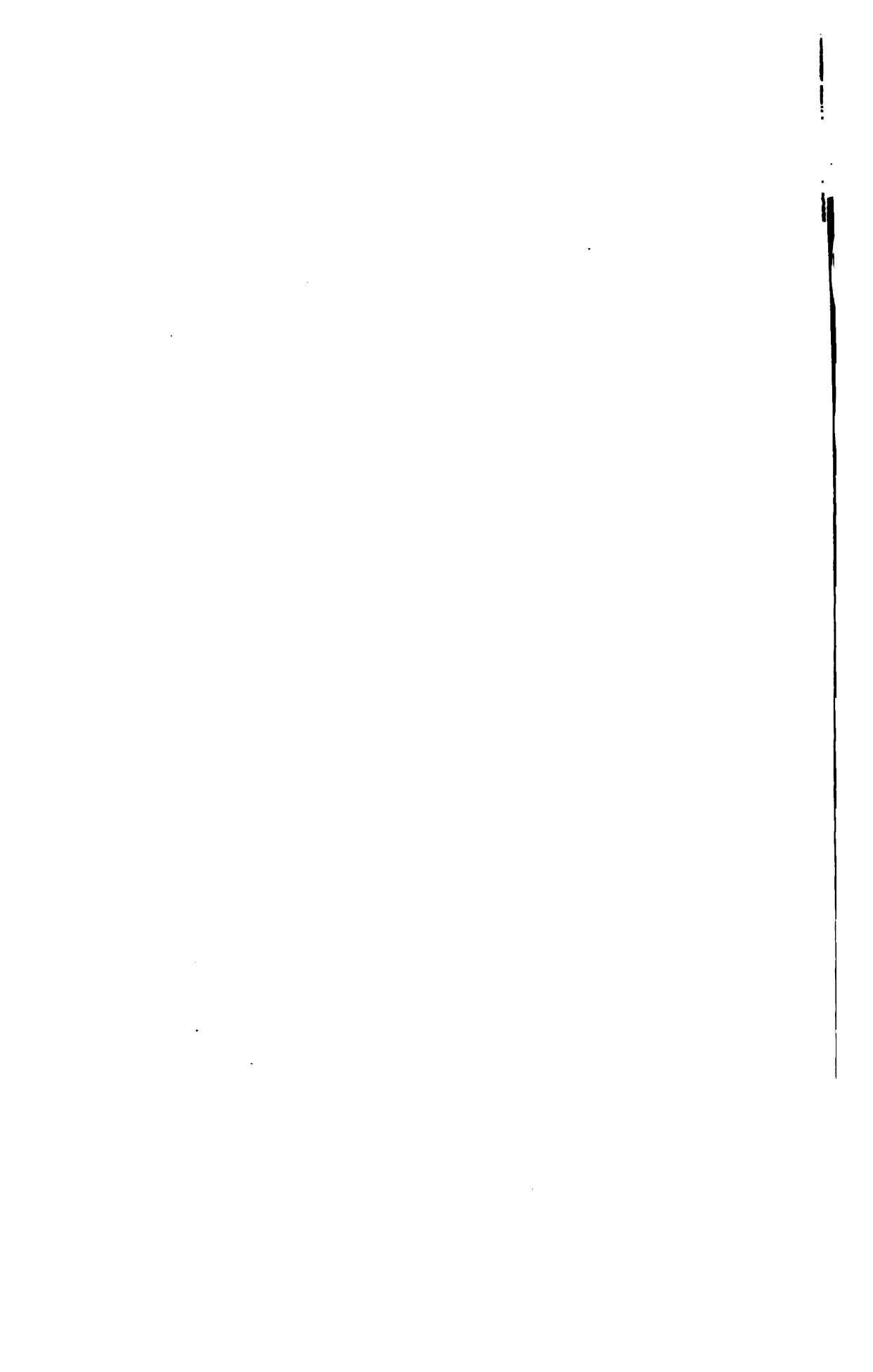


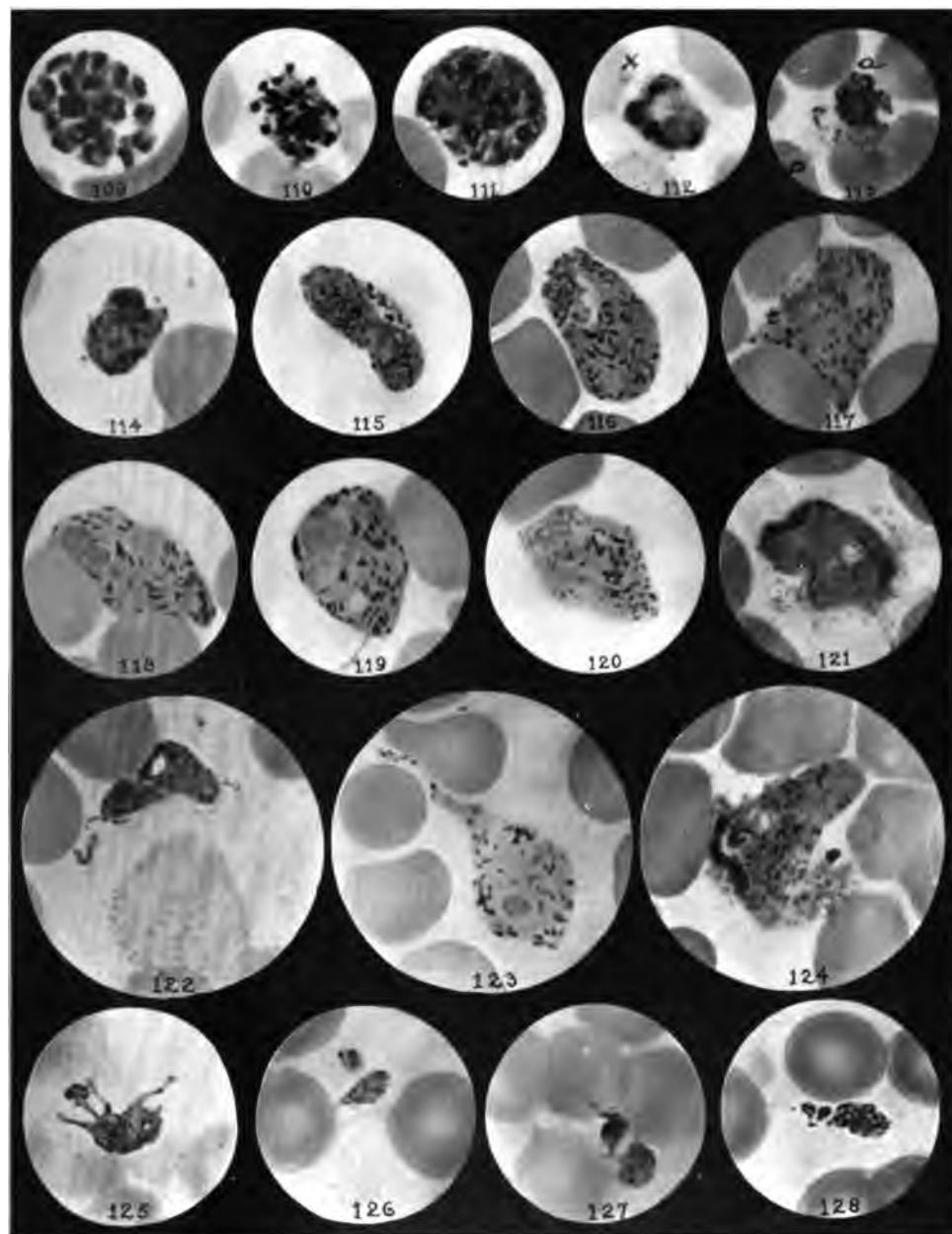
(Lawson: Free Malarial Parasites.)



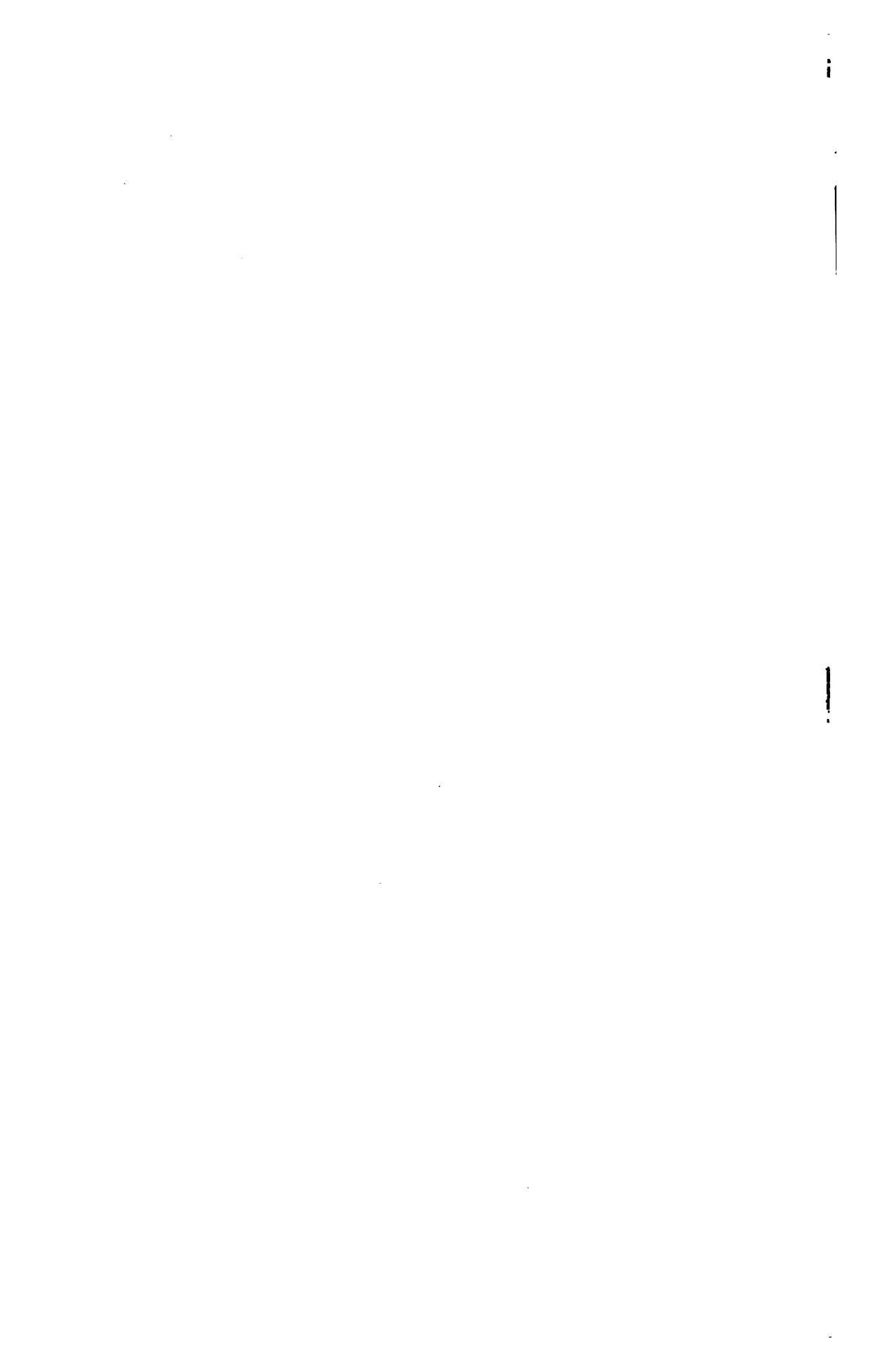


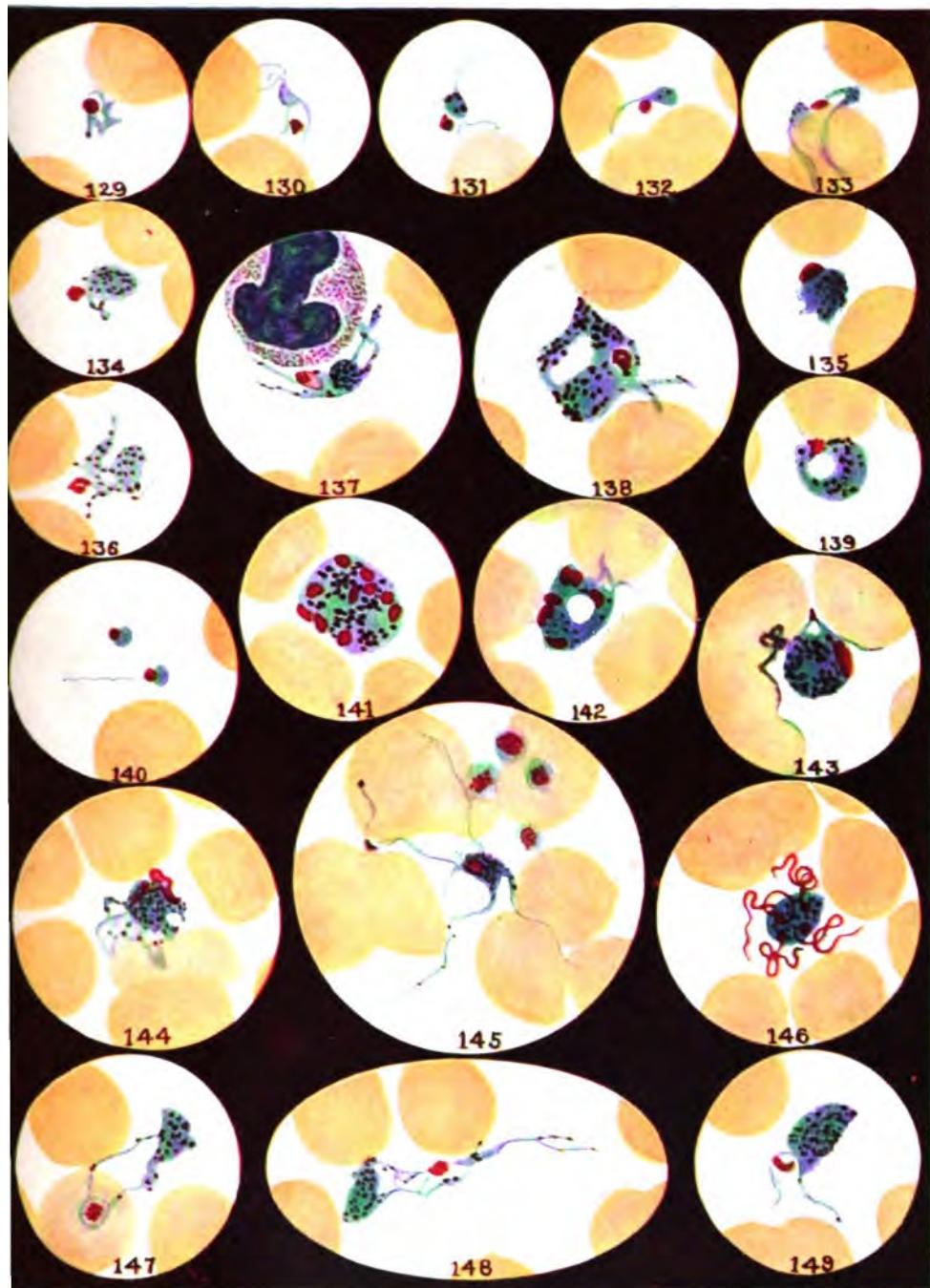
(Lawson: Free Malarial Parasites.)





(Lawson: Free Malarial Parasites.)





(Lawson: Free Malaria Parasites.)



## PLATE 66.

## FREE TERTIAN MALARIAL PARASITES.

FIGS. 129 to 133. (Correspond to figures 43, 42, 45, 37, and 44.) Young pigmented parasites with protoplasmic pseudopodia. In figure 133 the parasite appears to be attaching itself to the red corpuscle.

FIG. 134. (Corresponds to figure 48.) A pigmented parasite, freed with the pseudopodia in the form of a loop, which had been used to encircle a corpuscular mound.

FIG. 135. (Corresponds to figure 20.) A compact pigmented parasite.

FIG. 136. (Corresponds to figure 75.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 137. (Corresponds to figure 102.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 138. (Corresponds to figure 100.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 139. (Corresponds to figure 7.) A pigmented ring-form parasite, freed in the form which it had assumed when encircling a corpuscular mound.

FIG. 140. (Corresponds to figure 96.) A segment resulting from a recent sporulation, with a pseudopodium. It is rare to find one of these young parasites with a pseudopodium.

FIG. 141. A free presegmenting parasite.

FIG. 142. (Corresponds to figure 112.) A prefagellating parasite (microgametocyte) with pseudopodia. The parasite has been freed in the ring-form which it had assumed when encircling a corpuscular mound. Compare this parasite with the presegmenting body.

FIG. 143. (Corresponds to figure 99.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 144. (Corresponds to figure 113.) A sexual flagellating parasite (microgametocyte) with flagellum composed of chromatin substance, and pseudopodia (attaching processes) composed of the cytoplasm of the parasite.

FIG. 145. (Corresponds to figure 95.) A pigmented parasite with pseudopodia (attaching processes) arising from the cytoplasm of the parasite. Pigment granules can be seen in connection with some of the filaments. The other bodies seen in the picture are blood plates.

FIG. 146. A sexual flagellating parasite (microgametocyte), the flagella being derived from the chromatin substance of the parasite. Compare this parasite with the parasites with attaching pseudopodia. The attaching pseudopodia arise always from the cytoplasm of the parasite.

FIG. 147. A pigmented parasite with the pseudopodia in the form of a loop, which formerly was used to surround a corpuscular mound.

FIG. 148. (Corresponds to figure 104.) A pigmented parasite with attaching pseudopodia arising from the cytoplasm of the parasite.

FIG. 149. (Corresponds to figure 92.) Pigmented parasite with attaching pseudopodia arising from the cytoplasm of the parasite.

## THE ADRENALIN INDEX OF THE SUPRARENAL GLANDS IN HEALTH AND DISEASE.\*

By V. P. W. SYDENSTRICKER, B. J. DELATOUR, AND  
G. H. WHIPPLE, M.D.

(*From the Hunterian Laboratory of Experimental Pathology, Johns Hopkins Medical School, Baltimore.*)

During the past two years three methods for the quantitative estimation of adrenalin have appeared. They are accurate and sensitive to minute quantities of adrenalin, much more so than any quantitative methods hitherto used. The method of Elliott consists in the blood pressure response of a decerebrate cat to intravenous injection, standard adrenalin solutions being used as controls. The method of Folin consists of a colorimetric reaction of adrenalin with a phosphotungstic acid reagent, the standard being a pure uric acid solution. The method of Seidell consists of a colorimetric reaction of adrenalin with manganese dioxide, with adrenalin and gland extract as a control. Seidell's method appeared after this series of observations had been completed and we cannot therefore report on its usefulness in this type of work.

The chromaffin tissue is thought to have an intimate association with adrenalin production and storage. This tissue gives a deep brown color when treated with a solution of potassium bichromate, and various studies dealing with this substance have been published.

Vincent reports a study of this chromaphil substance in many animals, particularly the dog and cat. He notes that the chromaphil substance is found in considerable abundance in the dog outside of the adrenal medulla. The abdominal chromaphil body is situated just in front of the aorta, usually below the level of the adrenals. It is incapable of maintaining life in the absence of the two adrenals. An extract of this tissue can cause a rise in blood pressure like that produced by an extract of adrenal medulla. He states with reason that this body must be taken into consideration in all interpretations of experiments in which dogs are used.

\* Received for publication, March 14, 1914.

Schur and Wiesel claim that prolonged narcosis with chloroform or ether in rabbits may cause disappearance of the adrenal chromaffin, followed by a rapid return to normal in about twelve hours. They note hypoplasia of the chromaffin tissue of the adrenal medulla in *status lymphaticus*. Schwarzwald confirms this last observation, but not the observations on animals after anesthesia. Hornowski claims that in man and animals one may note a quantitative diminution of the adrenal chromaffin, depending upon the duration of anesthesia. He suggests that shock may depend in part on this depletion of the medulla chromaffin, which is concerned in the secretion of adrenalin.

Borberg has done an extensive series of experiments to show fluctuations in the chromaffin tissue under various experimental conditions. He used the chromate reaction and states that the chromaffin tissue coloration and adrenalin content vary with absolute parallelism, the one perhaps being the parent substance of the other. Inanition and asphyxia give a loss of chromaffin reaction; anemia may show a normal reaction; and intestinal obstruction shows a paler one. Chloroform anesthesia of short duration gives no change, but long anesthesia (three to seven hours) gives a marked weakening of the chromaffin staining. Ether or urethane anesthesia causes no change. Phosphorus poisoning gives a slight lowering of the chromaffin staining. He finds no indication by this method of hyperplasia of one adrenal after removal of the other gland. There is no reasonable doubt that the methods used in our work are much more delicate than the chromaffin reaction. If it is justifiable to argue concerning the adrenalin content from the density of the chromaffin reaction, surely one can reverse the process and assume that a more accurate estimation of adrenalin may give evidence concerning the depletion of the chromaffin tissue.

Elliott states that the chromaffin tissue diminishes in amount as the adrenalin content falls, but that no accurate determination is possible. His experiments deal chiefly with the adrenal glands of cats under various experimental conditions. He states that anesthesia by chloroform, ether, or urethane will cause a drop in the adrenalin content of the glands. Stimulation of sensory nerves or injury to the brain will cause a definite fall in the adrenalin content of the glands. He believes that drugs, stimuli, and trauma act through the central nervous system and that the impulses travel along the splanchnics.

#### METHOD.

A great many observations have been discarded because of inexplicable variations or slips in technique. This applies especially to the physiological method, as it will be found that some cats are refractory and give variable reactions to injections.

The glands from the human cases were removed at autopsy, and if not to be used immediately were placed on ice. They were usually cleaned and dissected carefully within a few hours of the autopsy, and the extract was made and tested. The adrenals were weighed and in many instances separate extracts made from the two glands (tables I to XVII).

The glands obtained from dogs were always fresh and removed at the end of an experiment or a few minutes after death. When not tested at once, the glands were wrapped in omental fat and placed on ice, but unless indicated in

the tables the extracts were made shortly after death. Experiments were done to indicate the influence of this preservation on ice and the rapidity of deterioration. It was found that gland *Brei* kept on ice for twenty-four to forty-eight hours would rarely show a diminution of 5 per cent. in adrenalin.

After dissection and weighing the glands were cut up and ground with sand to a uniform paste. This is a time-consuming process, but the determinations will not be uniform and dependable unless it is thoroughly carried out. The paste is then made up to a 20 per cent. solution with 0.9 per cent. salt solution, ground, and mixed thoroughly. After the sand has settled, the milky fluid is decanted and used as follows:

A. 5 c.c. of the 20 per cent. extract are combined with 5 c.c. of N/5 hydrochloric acid and heated to boiling. Sodium acetate (10 per cent.) is added drop by drop to the boiling solution until coagulation is complete. The mixture is diluted to 50 c.c. and filtered through paper giving a water-clear solution which corresponds to the solution used by Folin. Of this clear extract 10 c.c. are pipetted into a 100 c.c. volumetric flask, and 2 c.c. of the uric acid reagent and 20 c.c. of a saturated solution of sodium carbonate are added. The solution is shaken, made up to the mark, and after standing a few minutes is read against the standard solution. It is often necessary to use more than 10 c.c. of the gland filtrate to give enough color for accurate reading.

The standard uric acid solution is made by placing 1 c.c. of a fresh solution of uric acid containing 1 mg. of Kahlbaum uric acid in a 100 c.c. volumetric flask. The uric acid reagent and sodium carbonate are added as before and the solution is made up to the mark. The readings must be made promptly in a standard colorimeter as the colors fade. Folin, Cannon, and Denis have determined that pure adrenalin gives three times as intense a color as pure uric acid, so that by a simple calculation the amount of adrenalin in milligrams per gram of gland may be calculated.

B. Another portion of gland extract (20 per cent.) is diluted for use in the physiological test. With normal human gland extract a 1 per cent. solution is most satisfactory, but at times further dilution may be necessary, or in other cases a 10 per cent. extract must be used to elicit a satisfactory reaction. With canine gland extract it may be necessary to dilute to 0.2 per cent., so that a 1 c.c. injection can be used without the reaction being too intense.

The method of Elliott was carried out as follows: Cats were anesthetized, a tracheotomy performed, both vagi cut, the brain destroyed, and the cord pithed to the midthoracic region. Artificial respiration was immediately started and continued throughout the experiment. The blood pressure was recorded from the common carotid, and the injections were made by means of a cannula in the femoral vein. An interval of twenty to thirty minutes was necessary after pithing the animal before the blood pressure falls to a constant level. Under such conditions the standard reaction may be obtained by 0.5 c.c. of adrenalin (Parke, Davis and Co.) in 1:100,000 dilution. The solutions are injected rapidly and followed by a little normal saline to rinse out the cannula. The reaction blood pressure curves may be compared and measured, giving the amount of adrenalin in the extracts compared with the standard solution, which can easily be estimated by the Folin method.

As stated above, some cats will not give a uniform reaction to pure adrenalin

or tissue extract given in equal or fractional repeated injections. These experiments must be rejected. A suitable interval between injections is imperative. Very often it will be found that the reaction to successive doses of pure adrenalin solution in equal amount will not be uniform. The curves will become higher until a maximum is reached when subsequent injections will give lower curves. It is important not to tax the system by overdoses of adrenalin, as the subsequent reactions to smaller doses will be much below the first reaction to such doses. It is unsafe to push the blood pressure much more than 60 mm. of mercury above the constant blood pressure level, as the cats may show breaks in the even rounded curve and subsequent reactions may be inaccurate. Our practice has been to make alternate injections of pure adrenalin solutions and unknown extract, using minimal amounts and increasing to an optimum reaction. The adjacent curves may then be compared and the amount of adrenalin computed.

#### EXPERIMENTAL OBSERVATIONS.

The following type experiment (table I) shows the effect of preservation and autolysis upon gland emulsions of normal pigs. The animals were killed by a blow on the head, bled at once, and dressed as usual. The gland hash was made at once and tested at intervals as indicated in the table. It is clear that the drop in the adrenal index is not very rapid during preservation on ice.

TABLE I.  
*Adrenalin Index. Chemical Method.*

Normal fresh pig adrenal extract.....	1.604 mg. per gm.
Normal fresh pig adrenal extract plus 1 mg. adrenalin solution	2.540 mg. per gm.
Preservation on ice for 2 dys.....	1.578 mg. per gm.
Preservation on ice for 4 dys.....	1.463 mg. per gm.
Preservation on ice for 6 dys.....	1.200 mg. per gm.
Preservation <sup>1</sup> on ice for 8 dys.....	0.506 mg. per gm.

TABLE II.  
*Normal Dogs.*

No. of animal.	Adrenalin in mg. per gm. of gland.	
	Chemical method.	Physiological method.
94	1.396	...
107	1.360	...
12-105	1.416	4.31
N	1.83	5.70
12-140	1.167	3.09
0-92	1.25	4.55

Table II shows the normal adrenalin index for healthy dogs. These dogs were killed by means of ether anesthesia for about five

<sup>1</sup> At this time an odor of putrefaction was evident.

minutes and rapid bleeding from the carotid. The wide divergence of the chemical and physiological determinations will be discussed later (tables XIV to XVII).

TABLE III.

*Fatal Shock. Dog Injected Intravenously with Poison Obtained from Closed Duodenal Loops.*

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
0-30	0.450		Death in 4 hrs.
0-38	0.043		Death in 6 + hrs. Autopsy 12 hrs. post mortem.
0-44	0.388		Death in 4 hrs.
0-52	0.345		Death in 4 hrs.
0-53	0.303		Death in 4 hrs.
0-56	0.433		Death in 4 hrs. Autopsy 12 hrs. post mortem.
0-56	0.433		Death in 4 hrs. Autopsy 12 hrs. post mortem.
0-74	0.378		Death in 8 hrs. Autopsy 12 hrs. post mortem.
0-63	0.925		Death in 24 hrs. Bronchopneumonia.
110	0.417		Death in 2 hrs.

Table III shows the constant low adrenalin index of dogs poisoned fatally with fluid obtained from closed duodenal loops. The details of these experiments have been published elsewhere (Whipple, Stone, and Bernheim). The dogs die with constant symptoms of fatal shock, low blood pressure, and temperature, vomiting, and diarrhea, with profound collapse. The adrenalin index of dogs dying in four hours is about one fourth of normal, indicating perhaps a depletion of the adrenalin in an effort to compensate the lowered blood pressure.

TABLE IV.  
*Intoxication. Dog.*

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
0-35	0.186		High intestinal obstruction. Death in 5 dys.
0-39	0.566		High intestinal obstruction. Death in 6 dys.
0-58	0.483		High intestinal obstruction. Death in 3 dys.
0-106	1.25	0.29	Closed duodenal loop. Killed on 3d dy.
0-100	1.083	1.02	Closed duodenal loop. Killed on 3d dy.
D-L	0.833	1.80	Closed duodenal loop. Death on 4th dy.
0-121	0.917	3.90	Closed duodenal loop. Killed on 3d dy.
0-66	0.511		General peritonitis. Loop rupture.
0-60	0.811		General peritonitis. Bile duct rupture.
0-68	0.566		Acute hemorrhagic pancreatitis. Bile injection.

Table IV confirms the experiments in table III and shows a constant low adrenalin index in the intoxication of intestinal obstruction and closed loop experiments. Acute pancreatitis and general peritonitis may give the same low index.

TABLE V.  
*Anesthesia and Liver Injury. Dog.*

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
C-1	0.761		Killed at the end of 2 hrs. Chloroform anesthesia.
C-2	0.783		Perfusion for 3 hrs. Ether anesthesia.
0-117	1.00		Chloroform for 2 hrs. Killed 24 hrs. later.
12-63	1.125	3.35	Chloroform for 2 hrs. Killed 48 hrs. later.
12-59	0.532		Phosphorus fatal in 18 hrs.
12-76	0.975		Phosphorus fatal on 3d dy.
12-80	1.156		Phosphorus fatal on 6th dy.
12-64	0.018		Phosphorus subcutaneously. <i>B. coli</i> intravenously.
0-111	0.667	1.49	Liver extirpation. Killed in 5 hrs.
12-87	0.633		Hydrazine. Fatal on 2d dy.
12-93	0.811		Hydrazine. Fatal on 3d dy.

Table V shows the effects of chloroform or ether upon the adrenalin index. There is a fairly rapid return to normal. It is interesting that various liver poisons cause a definite fall in the adrenalin index. The more acute the injury the more pronounced is the diminution in adrenalin. The combination of a liver poison with a septicemia causes the most complete depletion of the adrenalin. This is true of chloroform as well as phosphorus, and such animals die in a few hours with symptoms of acute shock.

TABLE VI.  
*Pancreatic Diabetes. Cat.*

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.		
1	1.26		Normal.
2	0.20		Death in 7 dys. Constant glycosuria.
3	0.27		Death in 8 dys. Constant glycosuria.

Table VI shows the effect of removal of the pancreas in cats. These two animals after operation showed constant glycosuria and

rapid loss of weight. This observation is of interest when compared with a human case of exophthalmic goitre (table VIII) with fatal intoxication showing a low adrenalin index; also compare with human diabetes with a low adrenalin index (table XI).

TABLE VII.  
*Miscellaneous Conditions. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3969	2	0.417	0.75	Cerebral hemorrhage. Blood pressure 140 mm.
3877	31	0.293	0.22	Cerebral hemorrhage. Blood pressure 170 mm.
3877		0.360	0.24	
3867	15	0.289		Ruptured aneurysm (thorax).
3867		0.286		
3878	3	0.493	0.40	Trauma (brain); bronchopneumonia.
3878		0.451	0.40	
3868	16	0.00		Tuberculous adrenal.
3868		0.733		Prostatitis and abscesses.
3916	4	0.867		Pernicious anemia.
3916		0.867		

Table VII brings out several interesting points. It may be assumed that the human cases that die suddenly from hemorrhage or trauma are approximately normal. These cases together with some others cited below indicate that human adrenals shortly after death in approximately normal adults show an adrenalin index of 0.35 to 0.50; that is, they contain 0.35 to 0.50 of a milligram of adrenalin per gram of gland, as estimated by the chemical method. Postmortem deterioration is probably not rapid when the bodies are kept in an ice box and there is no evidence of autolysis. Human glands (right and left) do not contain exactly the same amounts of adrenalin per gram of weight, but contain approximately equal amounts.

It is noteworthy that one case (No. 3868) with a caseous adrenal (adrenalin index 0) showed almost double the normal amount of adrenalin in the other gland which did not show marked hyperplasia.

The case of pernicious anemia is of interest because of recent investigations which indicate that the spleen and adrenal may be concerned in lipoid metabolism which is thought to be upset in this disease. Physiological overactivity shows an increase in the adrenalin index of the suprarenal. May we argue the converse, that a very high adrenalin index means an increased activity of the gland? If the gland cortex is overactive, it is possible that the medulla may be stimulated to a somewhat parallel overactivity. By contrast it is seen that secondary anemias show a low adrenalin index (tables X and XI).

TABLE VIII.  
*Intoxications. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3883	8	0.471		Eclampsia.
3883		0.327		
3965	24	—	0.111	Exophthalmic goitre, toxemia.
3881	24	0.157		Baby 16 mos. Rachitis.
3908	18	0.162		Baby 11 mos. Rachitis.
3908		0.161		

Table VIII shows a case of eclampsia with the characteristic liver lesion, which died with very acute symptoms of intoxication, to have a normal adrenalin index. Cases of severe rachitis show a low index. Exophthalmic goitre with acute intoxication shows a very low adrenalin index. It is unfortunate that a chemical determination was not done, but it is probable from experiments done at this time under similar conditions that the chemical test would have been lower than the physiological, perhaps one half or one third.

Table IX shows a considerable variety of acute infections. It is seen that some cases of general peritonitis, meningitis, and typhoid may show a normal adrenalin index. The average is somewhat subnormal, but the drop below normal is not marked, and we may conclude that acute infections may not cause a great depletion of adrenalin stored in the glands.

TABLE IX.  
*Acute Infections. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3905	16	0.353		Peritonitis, pericarditis, pleurisy, and bronchopneumonia.
3905		0.142		
3896	14	0.173		General peritonitis.
3896		0.175		
3863	9	0.473		General peritonitis.
3863		0.510		
3862	24	0.280		Baby 6 mos. Pneumonia, meningitis.
3866	4	0.245		Bronchopneumonia.
3866		0.205		
3872	7	0.184	0.44	Baby 16 mos. Bronchopneumonia.
3872		0.181	0.15	
3871	24	0.330		Meningitis, bronchopneumonia.
3871		0.286		
3875	4	0.415	1.00	Otitis media, meningitis.
3875		0.375	1.11	
3917	14	0.252	0.43	Endometritis, septicemia, icterus.
3917		0.231	0.32	
3939	4	0.157		Abortion, suppurative nephritis.
3899	6	0.182		Pylephlebitis, icterus.
3899		0.211		
3911	10	0.434		Typhoid fever, toxemia.
3911		0.625		

It may be noted that two glands (No. 3872) gave equal amounts of adrenalin by the chemical method, but three times the amount in one gland compared with the other by the physiological method. There was no anatomical difference in the two glands and we are inclined to believe that the chemical method was more nearly correct.

Table X shows that in acute tuberculosis the adrenalin index may fall to about one half normal. It will be noted that the glands on the two sides may vary considerably as to their adrenal content. The last case shows one half as much adrenalin per gram in one gland as in the other.

TABLE X.  
*Tuberculosis. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.	Remarks.
		Chemical method.	
3891	15	0.220	Baby 22 mos. Miliary tuberculosis.
3891		0.249	
3898	16	0.250	Baby 7 mos. Miliary tuberculosis.
3898		0.330	
3924	15	0.058	Miliary and chronic tuberculosis.
3861	14	0.132	Pulmonary tuberculosis and ruptured aneurysm.
3861		0.287	

Table XI shows that the cachexia due to malignant tumors is associated with a low adrenalin index,—one half normal or even considerably less.

TABLE XI.  
*Neoplasms. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3903	1	0.205		Cancer of stomach, metastases.
3903		0.269		
3912	20	0.111		Cancer of esophagus, empyema.
3912		0.111		
3972	3	0.216	0.55	Cancer of prostate, suppurative nephritis.
3913	18	0.042		Cancer of stomach, general peritonitis.
3913		0.099		
3901	9	0.133		Hypernephroma, anemia.
3901		0.150		
3874	60	0.004	0.02	Adenoma of adrenal.
3874		0.012	0.20	Diabetes. Blood pressure 90 mm.

The case of malignant adenoma of the adrenal associated with diabetes is of especial interest. The diseased adrenal showed only a trace of adrenalin and the uninvolved gland showed a very low adrenalin index. We cannot attribute this solely to the post-mor-

tem change, as other autopsies of equal age show only a moderate fall below normal (tables XII and XIII). This observation harmonizes with those of table VI in which experimental pancreatic diabetes was associated with a low adrenalin index.

TABLE XII.

*Nephritis. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3865	50	0.261		Chronic nephritis, large kidney.
3865		0.260		Blood pressure 200 mm.
3935	8	0.522		Chronic and acute nephritis.
3935		0.405		Bronchopneumonia, pericarditis. Blood pressure 165 mm.
3882	6	0.182	0.50	Chronic nephritis. Blood pressure 190 mm.
3882		0.240	0.40	Acute endocarditis.

TABLE XIII.

*Cardiac Failure and Passive Congestion. Man.*

Case No.	Hrs. post mortem.	Adrenalin in mg. per gm. of gland.		Remarks.
		Chemical method.	Physiological method.	
3970	56	0.162		Myocarditis, pericarditis.
3897	18	0.368		Myocarditis, anasarca.
3897		0.371		
3909	4	0.091		Myocarditis, thrombosis.
3909		0.087		
3973	8	0.333	0.48	Myocarditis, bronchopneumonia. Blood pressure 175.
3869	4	0.495		Mitral insufficiency, Pott's disease.
3869		0.450		

Tables XII and XIII show that increased vascular tension associated with renal, cardiac, or vascular disease does not cause any great fluctuation in the adrenalin index. If anything the values are somewhat subnormal, but this may be accounted for in some instances by the secondary infections.

## METHOD CONTROL EXPERIMENTS.

Because of peculiar divergencies between the chemical and physiological determinations in some of the glands, group experiments were done in which as many unequal factors as possible might be eliminated. Our results do not agree with the series of observations of Folin, Cannon, and Denis in which the two methods agreed with startling uniformity. For this reason we were careful to eliminate as many sources of error as possible. In the following groups of experiments care was taken not to overtax the decerebrate organism by too great doses of adrenalin. The chemical determinations were done in duplicate and correspond roughly to the theoretical values. The physiological values at times are high and again low.

TABLE XIV.

No.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
12-140 (dog)	1.167	3.09-3.10	Normal.
0-92 (dog)	1.250	4.00-5.10	Normal.
0-117 (dog)	1.000	3.33-3.40	Chloroform anesthesia for 2 hrs. Killed 24 hrs. later.
.			
0-119 (dog)	0.917	1.88-2.55	Tissue juices intravenously. Death in 10 hrs.
0-111 (dog)	0.667	1.26-1.73	Liver extirpation—ether. Killed at end of 5 hrs.
3972 (man)	0.216	0.43-0.64	Cancer of prostate. Suppurative nephritis.
3973 (man)	0.333	0.36-0.60	Myocarditis. Passive congestion. Bronchopneumonia. Blood pressure 175.

Table XIV shows a group of observations done at the same time under the same conditions, using the same reagents and decerebrate cat. The chemical adrenalin indices vary from the normal dog down through lower values in the dog due to anesthesia, to the human cases of low value. Two series of physiological tests were done and are arranged side by side in the same table. Some determinations correspond and others do not, showing a variation of almost 50 per cent. The physiological determination is much higher than the chemical but the ratio is not the same in the different glands.

TABLE XV.

*Dog.*

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
N	1.83	5.70	Normal.
12-41	2.17	6.60	Recovery in 3 wks. following chloroform poisoning.
0-112	2.17	6.30	Dog immunized to duodenal loop poison.
0-121	0.92	3.90	Closed duodenal loop. Killed on 3d dy.
0-115	1.25	4.00	Dog immunized to duodenal loop fluid.
D-L	0.83	1.80	Closed duodenal loop. Death on 4th dy.

Table XV also shows a group of observations done at the same time under identical conditions. Again the chemical values correspond relatively to the theoretical ratio, which might obtain between the individual cases. These are the highest physiological values which we have ever observed, and this decerebrate cat was very sensitive to minute doses of adrenalin. It is interesting to compare this table with table XVII, where the physiological is below the chemical adrenalin index, in one instance of one fourth value.

TABLE XVI.

No.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
12-105 (dog)	1.42	4.31	Normal.
0-109 (dog)	1.67	2.62	Duodenal loop poison (sublethal dose). Recovery in 36 hrs. Killed.
3960 (man)	0.42	0.75	Cerebral hemorrhage. Blood pressure 140 mm.

Table XVI shows another group of experiments done on the same day under identical conditions. The second experiment (dog 0-109) shows a higher adrenalin index by the chemical method, but by the physiological method about one half the normal control.

Table XVII shows two groups of experiments done on successive days under identical conditions. It will be seen that the physiological method gives an index below that given by the chemical method. The values by the chemical method are in harmony with other observations in similar experimental animals given above. This illus-

TABLE XVII.  
*Dog.*

No. of animal.	Adrenalin in mg. per gm. of gland.		Remarks.
	Chemical method.	Physiological method.	
July 22			
0-106	1.25	0.29	Closed duodenal loop. Killed on 3d dy.
0-100	1.08	1.02	Closed duodenal loop. Killed on 3d dy.
0-102	0.75	0.63	Uremia(?)
July 23			
0-106	0.83		Solutions kept for 24 hrs. in cool place, not on ice.
0-100	0.67		
0-102	0.50		
0-110	0.42		Shock. Duodenal loop poison intravenously. Death in 2 hrs.
0-99	0.96		Immune dog with closed duodenal loop. Killed on 8th dy.

brates the wide fluctuations which may be observed in group experiments with the decerebrate cat. The last half of the table shows the rapid deterioration which may occur in the boiled filtered gland extracts kept in a cool dark place.

#### DISCUSSION.

The method of determining the adrenalin index, that is, milligrams of adrenalin per gram of gland, is open to error. In our hands the chemical colorimetric method has been much more uniform and subject to few sources of error. To the inexperienced worker the physiological method (with the decerebrate cat) is vastly more difficult than the chemical method. Different cats will not react in the same way, or they may react in the same way to pure adrenalin and in a different manner to the gland extracts. The tissue extract seems to affect different animals in a strikingly different manner. There are so many possibilities of individual variation among these cats as to cast doubt on any single series of observations with one animal. In our work if the cat had been given an overdose of adrenalin, perhaps causing only a rise of 60 to 80 millimeters of mercury, the subsequent reactions might be quite variable. The chemical method gives results which are more in harmony with the theoretical values, and known dilutions of a gland extract can be determined much more accurately than by the

physiological method. The same holds true for standardized gland extract, to a portion of which has been added a known amount of pure adrenalin. When the gland extract shows evidence of autolysis and a known amount of adrenalin has been added to a portion, it will be found that both methods may fail to estimate the adrenalin accurately. Tissue extracts in general tend to depress blood pressure, particularly products of autolysis, and this may explain the low results in some of the physiological determinations. It is possible that the adrenals contain some substance other than adrenalin which will cause elevation of blood pressure. It is difficult to explain some of the findings in the last few tables unless some such possibility is taken into account.

#### SUMMARY.

The adrenalin index as used in this paper means the amount of adrenalin in milligrams per gram of gland. As in our hands the chemical colorimetric method has proved more accurate, these values rather than the physiological values will be given in the final analysis. The two adrenal glands in the same individual as a rule contain about the same amount of adrenalin per gram, but variations of 10 to 20 per cent. are not unusual.

Normal dogs show an index which may vary from 1.2 to 1.8 milligrams. The dogs were killed by short ether anesthesia and bleeding from the carotid.

Normal human beings, dying from trauma, rupture of aneurysm, etc., show an index of 0.35 to 0.50 of a milligram, when autopsy takes place a few hours after death. Deterioration of uncut glands or of a gland hash kept on ice in the dark is not rapid and rarely exceeds 10 per cent. in twenty-four hours.

Acute intoxication in dogs shows a low adrenalin index, especially the intoxication associated with intestinal obstruction and the closed intestinal loop. Intravenous injection of the poison found in closed duodenal loops sufficient to cause fatal shock causes a great drop in the adrenalin index, at times to one fourth normal or even lower.

After recovery from a sublethal toxic dose the adrenalin index may rise rapidly to a point considerably above normal. The same may hold for recovery after chloroform poisoning.

Anesthesia by chloroform or ether causes a drop in the adrenalin index depending upon the length of anesthesia and probably in part on the depth of anesthesia.

Liver poisons (chloroform, phosphorus, hydrazine) cause a drop in the adrenal index to a low level, perhaps one half normal in acute cases.

Pancreas extirpation with prolonged glycosuria and death produces a great drop in the adrenalin index (cat). There is evidence that this may hold in some cases of human diabetes.

In man disease of one adrenal (tuberculosis) may be associated with an adrenalin index of double the normal value in the intact adrenal.

Pernicious anemia is the only disease so far found to present an abnormally high adrenalin index, and the single case shows an index at least twice normal. This is of interest especially in relation to the views recently put forward to indicate that the spleen and adrenal may be concerned in the lipoid metabolism which is thought to be profoundly disturbed in this disease.

Secondary anemia due to repeated hemorrhage or the intoxication of cancer or tuberculosis causes a fall in the adrenalin index. Cachexia due to neoplasm or tuberculosis may cause a marked fall in the adrenalin index, perhaps to less than one half of normal.

Acute infections (typhoid fever), septicemia, peritonitis, and similar conditions may be associated with a normal adrenalin index or one somewhat below normal.

Diseases of the kidneys, heart, or blood vessels associated with elevated blood pressure show no constant variation in the adrenalin index, which may be normal or slightly subnormal.

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## OVARIAN INFECTION IN THE DOMESTIC FOWL AND DIRECT TRANSMISSION OF DISEASE TO THE OFFSPRING.\*

By LEO F. RETTGER, PH.D.

(From the *Sheffield Scientific School of Yale University, New Haven.*)

In an extended discussion of the subject of inheritance and disease Adami<sup>1</sup> concludes that "specific infections are not inherited, but are of post-conceptional acquirement." Von Wassermann<sup>2</sup> holds the same opinion; in fact, this appears to be the generally accepted view. In the scientific interpretation of the term there can be no inheritance of disease. Parental properties are conveyed by the nuclear material of the spermatozoon and ovum; in other words, the inheritance of physiological and other properties is a function of the chromatin of the sperm and the egg. If germinal infection takes place and affects the offspring, the acquirement of the disease is by transmission only. Even this mode of handing transmission is disputed, however.

Instances of prenatal infection are too numerous to permit of doubt as to its occurrence, but it has at all times been difficult to exclude the possibility of intra-uterine infection, either as the result of a diseased condition of the placenta, or the passage of pathogenic organisms by way of the vagina through the amnion into the amniotic fluid. Syphilis is a common type of congenital disease, whereas tuberculosis appears to be transmitted rarely.

Calmette<sup>3</sup> claims that both placental and germinal transmission of tuberculosis are of uncommon occurrence in man. This view is strongly opposed by Baumgarten. In reviewing the literature Kerscher<sup>4</sup> found records of 181 alleged

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<sup>1</sup> Adami, J. G., in Osler, W., and McCrae, T., *A System of Medicine*, London, 1907, i, 17.

<sup>2</sup> von Wassermann, A., and Keysser, Fr., in Kolle-Wassermann, *Handbuch der pathogenen Mikroorganismen*, 2d edition, Jena, 1912, i, 659.

<sup>3</sup> Calmette, A., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 771.

<sup>4</sup> Kerscher, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1910, xxvi, 507.

cases of congenital tuberculosis in man. Of this number 55 appeared to him to be well authenticated. The instances of placental infection were estimated at 35. Landouzy<sup>5</sup> believes that the danger of congenital transmission from the mother is greater than from the father. Gaertner<sup>6</sup> cites a number of cases of congenital tuberculosis which are apparently of placental origin. Katholický<sup>7</sup> states that inherited tuberculosis is not rare.

Numerous instances of placental transmission of disease, besides syphilis, tuberculosis, and leprosy, are recorded;<sup>8</sup> for example, anthrax (Paltauf), pneumonia (Levy, Netter, Viti), typhoid fever (Eberth, Ernst, Freund, Levy), pyogenic coccus infection (Auche, Lebedeff, Fraenkel), recurring fever (Spitz), and variola (Champ). Scarlet fever, measles, malaria, articular rheumatism, glanders, and Asiatic cholera are claimed to have been transmitted in this way, also.

As to germinal infection, particularly in man, there has been less conclusive evidence. Baumgarten has for many years held the view that germinal transmission plays an important part in congenital tuberculosis. His views have been accepted by other investigators, but have not been substantiated by scientific observation. The instances of apparent ovarian infection in tuberculous women, and in guinea pigs that were experimentally infected with tubercle bacilli, have been too few and too uncertain to allow positive conclusions to be drawn from them.

Rosinski<sup>9</sup> strongly adheres to the theory of germinal transmission of syphilis, and claims to have demonstrated the certainty of paternal transmission through the spermatozoa. In as far as ovular infection is concerned, the egg may become infected, at the time of its development and great physiological activity, by organisms which may have been latent for some time. Neumann<sup>10</sup> also accepts the idea of ovarian transmission of syphilis to the offspring, and believes it is more common than the placental. The unprotected ovum appears to him to be more liable to infection than the fetus which is enclosed in the placenta. He furthermore demonstrates paternal transmission of disease through the sperm cells.

Sibley<sup>11</sup> reported an incident which has considerable bearing on the question of germinal infection. Eggs for hatching purposes were exchanged between two farmers. One of the farms had apparently always been free from avian tuberculosis. Soon after the introduction of the new stock (exchanged eggs) tuberculosis appeared in acute form.

Maffucci<sup>12</sup> inoculated eighteen eggs with a broth culture of the avian tubercle bacillus. After natural incubation of these eggs eight chicks were obtained.

<sup>5</sup> Landouzy, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1910, xxvi, 508.

<sup>6</sup> Gaertner, *Ztschr. f. Hyg. u. Infectionskrankh.*, 1893, xiii, 131.

<sup>7</sup> Katholický, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1903, xix, 476.

<sup>8</sup> Cited by von Wassermann, A., and Keysser, Fr., *loc. cit.*

<sup>9</sup> Rosinski, abstracted in *Jahresber. ü. d. Fortschr. in d. Lehre v. d. path. Mikroorgan.*, 1904, xx, 690.

<sup>10</sup> Neumann, *Wien. klin. Wchnschr.*, 1904, xvii, 551.

<sup>11</sup> Sibley, cited by Gaertner, *loc. cit.*

<sup>12</sup> Maffucci, A., *Centralbl. f. Bakteriol.*, 1889, v, 237.

All but one of the chicks were active, but small and delicate; one died thirty-six hours after hatching, without indications of tubercular infection. The others died after periods varying from twenty days to four and a half months. At least six out of the eight chicks showed marks of tubercular infection, and in several of these the presence of tubercle bacilli was demonstrated. Baumgarten<sup>18</sup> inoculated twelve eggs with tubercle bacilli. Only two chicks were obtained from these eggs, but both gave evidence of tuberculosis.

Gaertner<sup>14</sup> injected tubercle bacilli of human origin into the peritoneal cavity of twelve female canary birds. All succumbed to tuberculosis. The contents of two of the nine eggs laid by these birds produced tuberculosis in guinea pigs, when injected into the peritoneal cavity. The remaining seven eggs gave apparently negative results.

These experiments strongly indicate that germinal infection and transmission can, and undoubtedly do, take place, at least in fowls. They are fully supported by the observations upon which this paper is based.

The investigations of Pasteur and others on pébrine have shown conclusively that infected silkworm eggs are a grave source of danger to the propagation of the silkworm, and that the selection of sound and uninfected eggs is necessary for the success of this industry. Little, if anything, seems to be known as to the manner in which the eggs become infected; whether in the process of formation, during the act of expulsion, or as the result of contact with infected matter after the eggs are laid.

There is also abundant evidence that the organism of Texas or Southern cattle fever, the piroplasma of Smith, is carried in the eggs of the tick (*Boophilus bovis*). Here again the mode of infection of the eggs has not been determined, but that eggs from ticks which harbor the specific piroplasma are important agents in the transmission of this cattle disease has been satisfactorily shown.<sup>15</sup>

#### EXPERIMENTAL.

The following report is based entirely on a series of extensive observations made by the writer and his associates upon ovarian infection in the domestic fowl. This study was part of a general investigation of the economically important disease known in all parts of this country as bacillary white diarrhea of young chicks.<sup>16</sup>

Bacillary white diarrhea of chicks is a poultry scourge which was practically unknown prior to 1900. In its common manifestations it affects only chicks which are less than five or six weeks old. The greatest mortality occurs during the first two weeks. The symp-

<sup>18</sup> Baumgarten, cited by von Wassermann, A., and Keysser, Fr., *loc. cit.*

<sup>14</sup> Gaertner, *loc. cit.*

<sup>15</sup> Smith, T., and Kilborne, F. L., *Annual Reports of the U. S. Bureau of Animal Industry*, 1891-92, Nos. 8 and 9, 45, 177.

<sup>16</sup> Rettger, L. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, December, 1909, No. 60, 33; April, 1911, No. 68, 279. Rettger, L. F., Kirkpatrick, W. F., and Stoneburn, F. H., *idem*, December, 1912, No. 74, 153.

toms are those of acute bowel trouble; namely, diarrhea, listlessness, loss of appetite, subnormal temperature, and extreme weakness, especially in the later stages.

One of the earliest known epidemics came to the writer's notice in the summer of 1899. During an investigation of this epidemic an organism was isolated from the liver, heart, and lungs of chicks which died from the disease. Inoculation of normal chicks with this organism resulted in the death of the chicks, the symptoms and post-mortem appearances being the same as those for typical bacillary white diarrhea. In the numerous epidemics which have been studied since that time the same organism has invariably been found. Its definite relation to the disease has been established repeatedly by inoculation and artificial feeding experiments. A brief description of this microorganism, now generally known as *Bacterium pullorum*, follows.

*Morphology, Cultural Characteristics, etc., of Bacterium pullorum.*—The organism is a long, slender bacillus (0.4 to 0.5  $\times$  2 to 4 micra) with slightly rounded ends. It usually occurs single, chains of more than two bacilli being rarely found. It is a non-motile, non-liquefying, non-chromogenic, facultative anaerobe. It is stained readily by the ordinary basic anilin dyes, but does not retain its stain by the Gram method. It does not produce spores.

*Agar Plates.*—Small colorless colonies make their appearance in twenty-four hours at 37° C. They increase in size slowly, and seldom attain more than one millimeter in diameter. They may be round, oval, or spindle-shaped. The surface is marked with one or two rosette figures. The border is comparatively smooth.

*Slant Agar.*—When streaked in the usual manner, a visible growth is obtained in twenty-four hours which spreads little, and remains delicate, even after three or four days of incubation. When, however, the entire surface of the agar is streaked with a platinum loop the characteristic cultural appearance of the pyogenic streptococcus is obtained, the growth being made up of minute discrete colonies which may be so small as to require a magnifying lens for their detection. This cultural characteristic is of extreme importance in identification work.

*Bouillon.*—The growth is like that of *B. typhi*, though as a rule not so luxuriant.

*Gas Production in Sugar Bouillon.*—Some of the strains produce gas in dextrose and mannite broth, whereas others lack this property. No reactions of any importance are obtained in any of the other laboratory sugar media.

*Indol and Nitrite Production.*—Neither indol nor nitrite could be detected in Dunham's peptone solution at the end of one week's incubation.

In its morphological, staining, and in certain of its cultural characteristics *B. pullorum* resembles *B. typhi*, and must, therefore, be classed as a member of the coli-typhi-enteritidis group of bacilli.<sup>17</sup>

Numerous feeding experiments in which the food was artificially infected with *Bacterium pullorum* demonstrated without any doubt that bacillary white diarrhea is highly infectious, and that it is transmitted from chick to chick through infected droppings. This fact furnishes an explanation of the rapid spread of the disease and its frequent occurrence in epidemic form. It does not, however, establish the immediate source of infection in those chicks which are the first to become affected.

#### THE OVARIES OF THE BREEDING STOCK AS THE IMMEDIATE SOURCE OF INFECTION.

One of the peculiar conditions that had frequently been observed in diseased chicks was the failure to absorb the yolk. In fact, this is one of the most striking symptoms of the disease, and makes itself manifest in the protrusion of the abdomen below the vent. Bacteriological examinations of the contents of the yolk sac invariably revealed the presence of *Bacterium pullorum* in the yolk.

It was but natural to assume that the yolk played an important part in bringing about a diseased condition of the chick while still in the egg. This assumption was further supported by the successful search for the bacterium in question in the yolks of infected chicks at the time of hatching, and of chicks at various stages of their development within the egg. Furthermore, when eggs which came from breeds of hens whose progeny had been known to be subject to white diarrhea were examined, *Bacterium pullorum* was frequently found. Not only eggs which were in various stages of incubation harbored the organism in the yolk, but perfectly fresh eggs as well.

It required but one more link in the chain of evidence to trace white diarrhea infection to the laying hen; namely, the demonstration of the presence of the organism in the ovary. This was ac-

<sup>17</sup> For a more detailed account of *B. pullorum*, see Rettger, L. F., and Harvey, S. C., *Jour. Med. Research*, 1908, xviii, 277; 1909, xxi, 115. Rettger, L. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, December, 1909, No. 60, 33.

complished without difficulty. Small flocks of hens, the eggs and chicks of which had revealed a history of white diarrhea infection, were killed and examined. Out of the first lot of 23 hens, 21 had abnormal ovaries which were found to contain *Bacterium pullorum* unaccompanied by any other organism.

A normal ovary which is undergoing natural development is made up of numerous ova of varying sizes. Some of the ova may be as large as an egg yolk; on the other hand, there are numerous minute ova many of which require magnification in order to be seen. The small ova are round and colorless, and may be likened to small tapioca grains that have been soaked in water. The color of the developing ova varies from a light yellow to a rich normal yolk color.

A typical infected ovary is composed of two distinct types of ova, the normal and the abnormal. The normal resemble those of perfect ovaries. The abnormal may vary as to size, and occasionally as many as twelve or more comparatively large ova, of practically uniform size, may be seen. The small cysts are usually less angular and irregular than the large ones. The color of the large cysts may vary greatly. Some are light, others dark; almost all shades of yellow and brown may be present. Occasionally the color suggests gangrene. The larger abnormal ova are usually quite angular in form, and of a firm consistency. At times they are so compressed as to appear flattened. The contents of the pathological ova are quite characteristic, consisting chiefly of a solid cheese-like matter which is permeated by a clear amber-colored fluid.

An infected ovary can be recognized readily, even by those who have had no special training. Hence, the direct examination of ovaries constitutes one of the best methods of determining the presence of white diarrhea infection in breeding stock. The bacteriological examination of the pathological ova is desirable, however, as a corroborative test.

#### CYCLE OF INFECTION.

Three possible explanations as to the manner in which ovarian infection with *Bacterium pullorum* is brought about naturally present themselves. First, chicks which have been victims of, but which have survived an attack of bacillary white diarrhea, may continue to harbor the organism of the disease for long periods of time, even to maturity, when the infection becomes localized in the ovary; second, fowls may become diseased by the ingestion of food and water which have been contaminated by the droppings of infected chicks, or carriers; and third, infection may be brought about by

association or contact with fowls which harbor the organism in question, as through the agency of body lice or of mites. There is also a possibility that the male may play some part in the transmission of the bacterium, although numerous attempts to demonstrate this have failed, and no evidence can be gathered that *Bacterium pullorum* is ever present in the testes.

It has been definitely shown that mature hens may acquire infection by feeding on food that has been artificially infected with broth cultures of *Bacterium pullorum*, and that the ovaries in particular become involved; but there is much doubt as to the probability that fowls which contain the organism in the ovum as the localized seat of injury eliminate the organism in the droppings, so that their excreta are a menace to other fowls. Although the question as to whether ovarian transmission from adult to adult hens takes place to an appreciable extent needs further elucidation, the carrying of infection to full maturity by chicks that survive the disease has been conclusively demonstrated in our investigations of the past year.

The experiments were conducted as follows.

A large number of chicks which were from twenty-four to forty-eight hours old were employed. They were divided into two lots, one of which was artificially infected with broth cultures of *B. pullorum*, while the other was not given this treatment, but served as a control for the first lot. In each experiment typical white diarrhea made itself felt throughout the artificially infected pens, with a high mortality, whereas the control pens remained unaffected. After the disease had fully run its course, or when the chicks were from seven to eight weeks old, the female chicks were transferred to permanent poultry houses where they were kept under daily observation until they were a year old, when the final examinations were made. The houses and the yards into which they opened had been thoroughly cleaned and disinfected, and had been left unoccupied for at least three or four weeks.

Of the 138 chicks which grew to maturity and lived until the termination of the experiments, 88 were known to have been infected with the organism in question, while the remaining 57 were survivors of the original controls. Of the 88 that were infected as chicks, 21 gave positive agglutination tests when they were about a year old, and showed unmistakable evidence of ovarian infection with *B. pullorum* at the time of autopsy. In other words, almost 25 per cent. of the chicks which were known to have been infected became bacillus carriers, the ovaries being apparently the only organs that were involved.

On the other hand, but one of the control lots showed any indications of ovarian infection, 56 of the 57 controls giving negative results in the agglutination tests and at the final examination. It is possible that the single exception may have suffered accidental infection as a chick or later in life.

Since the female chicks had been separated from the males at an early age (seven to eight weeks), and as no males were kept in the permanent houses, it may be concluded that the ovarian infection was brought about as the result of early infection of the chicks; hence our evidence as to a cycle of infection in bacillary white diarrhea is complete. The original source of infection is the ovary of the mother hen. The infected ovary produces eggs which carry the bacterium of white diarrhea in them; consequently chicks which are developed in these eggs are infected at the time of hatching. The infected chicks become an immediate source of danger to other or normal chicks, and epidemics of white diarrhea take place. Surviving female chicks become permanent bacillus carriers, and when mature they harbor the organism of the disease permanently in the ovary.

Numerous ovaries that have been examined by us have been so badly diseased in the manner described that the production of normal egg yolks had probably long ceased. On the other hand, there is abundant evidence that fowls which possess pathological ova continue laying through an entire laying season or longer. It is probable, too, that the most active layers are the most susceptible to ovarian infection, since the physiological activity of the ovary is such as to lessen its vitality and make it an easy prey to an organism which attacks the ovary more easily than any other organ. On the other hand, an ovary which is comparatively inactive or dormant is less apt to be disturbed.

As a rule, only a comparatively small number of the eggs that are laid by fowls having abnormal ovaries contain the organism of bacillary white diarrhea. It is often necessary to examine as many as eight or ten eggs from a single fowl before any positive evidence may be obtained by this method as to ovarian infection, although the ovaries are decidedly involved. This fact greatly lessens the value of routine egg-testing as a diagnostic measure, especially in as far as selection of individual fowls for breeding is concerned. As an aid in determining ovarian infection in flocks of fowl, however, it is of much importance.

The comparative infrequency with which *Bacterium pullorum* is, as a rule, transmitted to the egg, even when the ovary has been gen-

erally affected by the organism, is but another illustration of the interesting fact that nature does her utmost to protect the offspring in its period of embryonic development.

It is also of particular interest to note that eggs which carry the bacterium in question in their yolks harbor the organism in such small numbers that it is practically impossible to detect it unless the entire yolk is employed in the examination, or unless the eggs have been incubated at body temperature for at least three or four days, or until multiplication of the organism within the yolk has taken place. It is certain that while the yolk is held within the ovary or the oviduct of the fowl there is little or no reproduction of *Bacterium pullorum*; it is only after the time of laying that the apparently inhibitive influence of the yolk on the bacterium is reduced or completely lost.

There is no apparent difference between infected and uninfected eggs, aside from the presence of *Bacterium pullorum* in the former, and this can be detected by cultural methods only. In shape, size, color, odor, taste, and consistency the yolks of eggs that contain the bacterium of bacillary white diarrhea can not be distinguished from those of normal eggs.

The contents of eggs that are produced by normal fowls are, with rare exceptions, sterile at the time of laying and for several weeks after, if the shells remain whole, dry, and clean. This has been conclusively demonstrated by the writer.<sup>18</sup> Furthermore, numerous bacteriological examinations have shown that ovaries which are not infected with *Bacterium pullorum*, and which appear to be normal, are invariably sterile. The oviduct has also been found to be sterile to within a distance of three or four inches of the cloaca. It may be said, therefore, that in the fowl nature ordinarily makes the necessary provisions for the safeguarding of the young against early invasion by microorganisms.

The claim is not made here that the demonstration of ovarian infection with *Bacterium pullorum* in fowls is proof that germinal transmission of disease in mammals, and especially in man, can or does take place; but it makes the possibility of such transmission in man all the more apparent. The mere fact that the ova of fowls

<sup>18</sup> Rettger, L. F., *Centralbl. f. Bakteriol., 2te Abt.*, 1914, xxxix, 611.

and mammals are different as to their structure and development is not sufficient evidence that bacterial invasion of the mammalian ovule is impossible or even improbable. The yolk or food substance in the egg of fowls is not necessary for the successful invasion and establishment of *Bacterium pullorum*, as all the evidence at hand indicates that this organism does not multiply, at least to any noticeable extent, in the ovum or yolk until long after the egg leaves the body, but that it lies dormant during the entire period of ovarian development, and even for a time after the egg is completed. These statements apply only to ova that develop normally and are expelled into the oviducts, and not to the pathological cysts that are retained in the ovary.

These observations are in accord with Baumgarten's view regarding germinal transmission as well as with the opinions of other investigators to whom reference has already been made.

#### SUMMARY.

Ovarian infection and germinal transmission of disease have been conclusively demonstrated in our investigations of bacillary white diarrhea in the common domestic fowl. The disease, which has caused so much loss to the poultry industry in recent years, primarily affects young chicks that are but a few weeks old.

Chicks which survive frequently become permanent bacillus carriers, the ovary being the important seat of infection. The eggs from such carriers often harbor the organism of the disease in the yolk. Chicks that develop in infected eggs become in turn infected, and have the disease at the time of hatching. The disease is transmitted to normal chicks through the infected droppings; thus an epidemic is produced, and the cycle of infection is completed.

There is no evidence to indicate that germinal transmission through the male takes place. In view of the frequent negative results bearing on this question it seems probable that it does not.

MORPHOLOGICAL AND DEVELOPMENTAL ANOMALIES OF A PATHOGENIC STRAIN OF TRYPANOSOMA LEWISI AND THEIR RELATION TO ITS VIRULENCE.\*

By WADE H. BROWN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 67 AND 68.

The occasional references to unusual manifestations of virulence and the numerous descriptions of morphological and developmental anomalies of *Trypanosoma lewisi* contain scarcely a suggestion of a connection existing between changes in the virulence and changes in the morphology and developmental tendencies of the organism. The first suggestion of the possibility of such a relation is contained in the work of Wendelstadt and Fellmer,<sup>1</sup> on the effect of the passage of *Trypanosoma lewisi* through cold-blooded animals. These authors noted an increase in the virulence of the trypanosome for rats, and, at the same time, a profound alteration in the morphology of the organism, the main feature of which was a marked prolongation of the posterior extremity.

In a recent article<sup>2</sup> I published an account of an unusual increase in the virulence of a pathogenic strain of *Trypanosoma lewisi*. As this strain showed many morphological and developmental peculiarities, the life cycle and morphology of the organism were studied in eighty-two rats, with especial reference to the nature of the infection. In different series of infections, the rate of passage and the dose of trypanosomes were varied in an attempt to modify the resulting infections. As a control to this series, a study was made of a parallel series of rats infected with a typical benign strain of

\* Received for publication, April 13, 1914.

<sup>1</sup> Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 422; 1910, v, 337.

<sup>2</sup> Brown, W. H., *Jour. Exper. Med.*, 1914, xix, 406.

*Trypanosoma lewisi* that showed no morphological or developmental peculiarities. The details of these experiments and the effect of such procedures, as indicated, upon the course of the infection will be presented in another paper.

The usual life cycle and morphology of *Trypanosoma lewisi* in the rat's blood are so well known that they require no description, and it is not my purpose to present an exhaustive study of the anomalies of this organism and the factors giving rise to them, but rather the relation of anomalous development and morphology to the virulence exhibited by this particular strain. Therefore, only those deviations from the normal will be described that were pronounced and occurred with sufficient frequency in both fresh and stained preparations to be regarded as characteristic of the strain or of a particular infection. Most, if not all, of the peculiarities of this strain have been recognized by other workers but some of them are comparatively rare and apparently have not all been recognized in a single strain, while the conditions giving rise to these anomalies are but little understood.

#### DEVELOPMENTAL ANOMALIES.

In addition to the usual forms of multiplication, two clearly differentiated types of longitudinal division were observed; both of these were examples of equal binary fission; in rare instances the division was unequal or was multiple. The first of these types of division (figures 1 and 2) is more closely related to the usual process of multiplication as it occurred in trypanosomes showing the increased size, granular and basophilic cytoplasm characteristic of this period in the life cycle of the organism. Such forms of division were never numerous and were observed only during the usual period of multiplication. The sequence in the process of division could not be determined with certainty, as our only means of recognizing these forms comes with the division of the flagellum and undulating membrane. It is certain that division of these structures may occur first, but it is impossible to say whether trypanosomes with a divided nucleus or blepharoplast may not ultimately divide in this manner.

The second type of longitudinal division (figures 3 to 7) occurred far more frequently than the first and was observed during all phases of the blood infection. These forms presented a great variety of sizes, occurring in organisms that were quite large as well as in exceedingly small ones, but the general contour, nature of the cytoplasm, and staining reactions of all were characteristic of adult trypanosomes. A study of a large series of these forms indicated that the usual sequence of division was flagellum, undulating membrane, nucleus, and blepharoplast; but again, the sequence was undoubtedly subject to variation.

Several examples of multiple longitudinal division of adult trypanosomes were seen, one of which is shown in figure 7, but unequal division of this type of organism was not observed.

From published accounts of longitudinal division of *Trypanosoma lewisi*, it is uncertain whether both of these types of division have been recognized; the second is undoubtedly the one that is generally described. In a sense, the two types might be regarded as but one, since they grade into each other and probably represent a common tendency initiated in the young trypanosome and persisting in the adult organism even after all other forms of multiplication have disappeared from the blood.

The almost endless variety of forms exhibited by *Trypanosoma lewisi* in the usual process of multiplication precludes any consideration of the morphology of such organisms except as regards the degree of irregularity. Further, during the period of multiplication variations in the rate, extent, and persistence of multiplication may occur which markedly alter the character of the infection. At least two types of deviation from the normal have been recognized as indicating an increased severity of infection; either multiplication may be very rapid and marked with great numbers of irregular forms, especially small and imperfectly developed trypanosomes, or, with a normal rate and type of multiplication, this period of the life cycle does not cease at the usual time but continues on through the second or even the third week of the infection. According to our experience, the first type of infection is typically acute and occasionally terminates fatally at the height of multiplication, while the second, although usually more severe, progresses more slowly, and

fatal terminations occur, as a rule, after the second week of the infection.

#### ATYPICAL MORPHOLOGY.

Adult forms of *Trypanosoma lewisi* are generally uniform in character. The variations that we noted concerned the size, the nature of the flagellum and undulating membrane, the character of the posterior extremity, and the nucleus and the blepharoplast of the trypanosome.

Extremely small trypanosomes, measuring no more than 7 to 8 micra from the tip of the posterior extremity to the origin of the free flagellum, but possessing all the other characteristics of the normal adult trypanosome, were numerous in some severe infections. These small trypanosomes (figures 8 and 9) are of especial interest, as they appear to be identical with the small forms regarded by Swellengrebel and Strickland<sup>3</sup> as peculiar to the life cycle of *Trypanosoma lewisi* in the invertebrate host.

The posterior elongated forms of *Trypanosoma lewisi* described by many authors, and erroneously regarded by some as "of constant occurrence and very numerous at a certain stage of the multiplication-period,"<sup>4</sup> were very numerous in this strain of the organism. In some instances 10 per cent. of the trypanosomes showed this peculiarity (figure 10). Two types of elongated extremity were observed with about equal frequency. One of these possessed a delicately pointed tip (figures 11 and 12), and the other a bulbous extremity (figures 3 and 13). These posterior elongated forms were observed during all periods of the infection, but were most numerous during the latter part and immediately following the multiplication period. While this variety of trypanosome was usually larger than the normal adult, extremely small and irregular forms, such as those in figures 14 and 15, were occasionally seen. Other peculiarities of structure that usually accompanied an elongation of the posterior extremity were a highly developed and plicated undulating membrane and a short free flagellum, both of which are shown typically in figure 11. In a few fortunately stained prepa-

<sup>3</sup> Strickland, C., and Swellengrebel, N. H., *Parasitol.*, 1910, iii, 436.

<sup>4</sup> Minchin, E. A., *An Introduction to the Study of the Protozoa*, London, 1912, 292.

rations of these organisms a series of extremely faint longitudinal striations were seen that suggested the presence of myonemes.

Trypanosomes with an abnormally short posterior extremity (figure 16) were not infrequently observed along with the elongated variety. Occasionally there was a virtual absence of the post-blepharoplasic segment, the blepharoplast being practically at the tip of the extremity.

The undulating membrane and the flagellum of *Trypanosoma lewisi* may vary quite independently of the posterior extremity. The two trypanosomes in figure 17 show extremes in the development of the undulating membrane, while figure 18 shows an organism in an early stage of division with an unusually prominent membrane. Figure 19 illustrates an extreme shortening of the free flagellum in an otherwise peculiar trypanosome.

Innumerable irregularities of the nucleus and blepharoplast have been described in detail by various authors and most of them are properly regarded as involution or degeneration phenomena. I observed total absence of a stainable nucleus, resulting from atypical division, in a few instances. This anomaly occurred in young trypanosomes with no evidence of degeneration, as well as in adult organisms where degeneration could not be excluded.

The production of ablepharoplasic trypanosomes through the agency of drugs has attracted so much attention that the spontaneous occurrence of this anomaly in *Trypanosoma lewisi* deserves especial mention. Figures 20 to 22 show ablepharoplasic trypanosomes that are either young or early multiplication forms with no evidence of degeneration or mechanical distortion. Absence of a blepharoplast was noted in all types of *Trypanosoma lewisi*, but was most frequent in the adult organism. While in most instances there was not the slightest suggestion of a blepharoplast, occasionally there was an extremely small granule (figure 20), representing a rudimentary blepharoplast. Although this type of organism was constant, and even numerous in many infections, it could not be found in other infections of the same strain of *Trypanosoma lewisi*. Examples of the developmental and morphological anomalies of this strain might be greatly extended, but these are sufficient to show the unusual character of the strain.

RELATION OF DEVELOPMENTAL AND MORPHOLOGICAL ANOMALIES  
TO VIRULENCE.

In studying these features of the organism with reference to the character of the infection, as indicated earlier in this paper, certain facts were brought out that indicated a degree of correspondence between the occurrence of developmental and morphological anomalies and the virulence of the strains with which I was working. As the blood of each of the eighty-two rats infected with our pathogenic strain of *Trypanosoma lewisi* showed most of the anomalies of development and morphology that have been described, these features may be regarded as characteristic of the strain. The control strain which, when first isolated, produced very benign infections and showed no anomalies, subsequently showed an increased virulence in certain series of infections coincident with the appearance of anomalous forms of trypanosomes in the blood of infected rats. The simultaneous alteration of these several properties of the organism suggests something more definite than a mere coincidence.

Further, in both strains the relative numbers and the variety of atypical trypanosomes differed in different infections and were greatest in two classes of infections, both of which have already been described as atypical infections. In one of these the incubation period was short, multiplication was rapid and pronounced, and there were enormous numbers of trypanosomes in the peripheral circulation. Some of these infections were unusually severe and occasionally terminated fatally, while others, in which multiplication ceased early and abruptly, showed a very mild course throughout.

The second class of infections in which anomalies were prominent differed from the first in that the evolution of the infection was more gradual and the period of multiplication persistent. The prominent features of these infections were the persistence of multiplication and the severity of the infection.

In conclusion, then, while it was certain that an intimate relation existed between the developmental tendencies and the morphology of these strains of *Trypanosoma lewisi*, it could not be definitely determined to what degree these characteristics were coördinated with the virulence. There were some facts that might lead one to believe

that as virulence influenced the course of the infections it also exercised an influence upon the developmental tendencies and hence the morphology of the organisms. My observations, however, inclined me to the opinion that, in so far as *Trypanosoma lewisi* was concerned, developmental tendencies exercised the dominant influence upon the morphology of the organism, and, while these characteristics did not correspond rigidly with the virulence of the organism, that anomalies of development and morphology, to some degree, corresponded with the virulence as manifested in the infections of *Trypanosoma lewisi* that I studied.

#### SUMMARY.

1. The morphological and developmental anomalies of a pathogenic strain of *Trypanosoma lewisi* have been described to show the unusual character of the strain.
2. Especial attention is called to the spontaneous occurrence of ablepharoplastid forms of *Trypanosoma lewisi*.
3. It is pointed out that morphological anomalies were most pronounced in infections that showed unusual conditions of multiplication, and that such infections usually proved severe.
4. Finally, an appreciable correlation between the morphological and developmental characteristics and the virulence as manifested in these examples of infection with *Trypanosoma lewisi* has been suggested.

#### EXPLANATION OF PLATES.

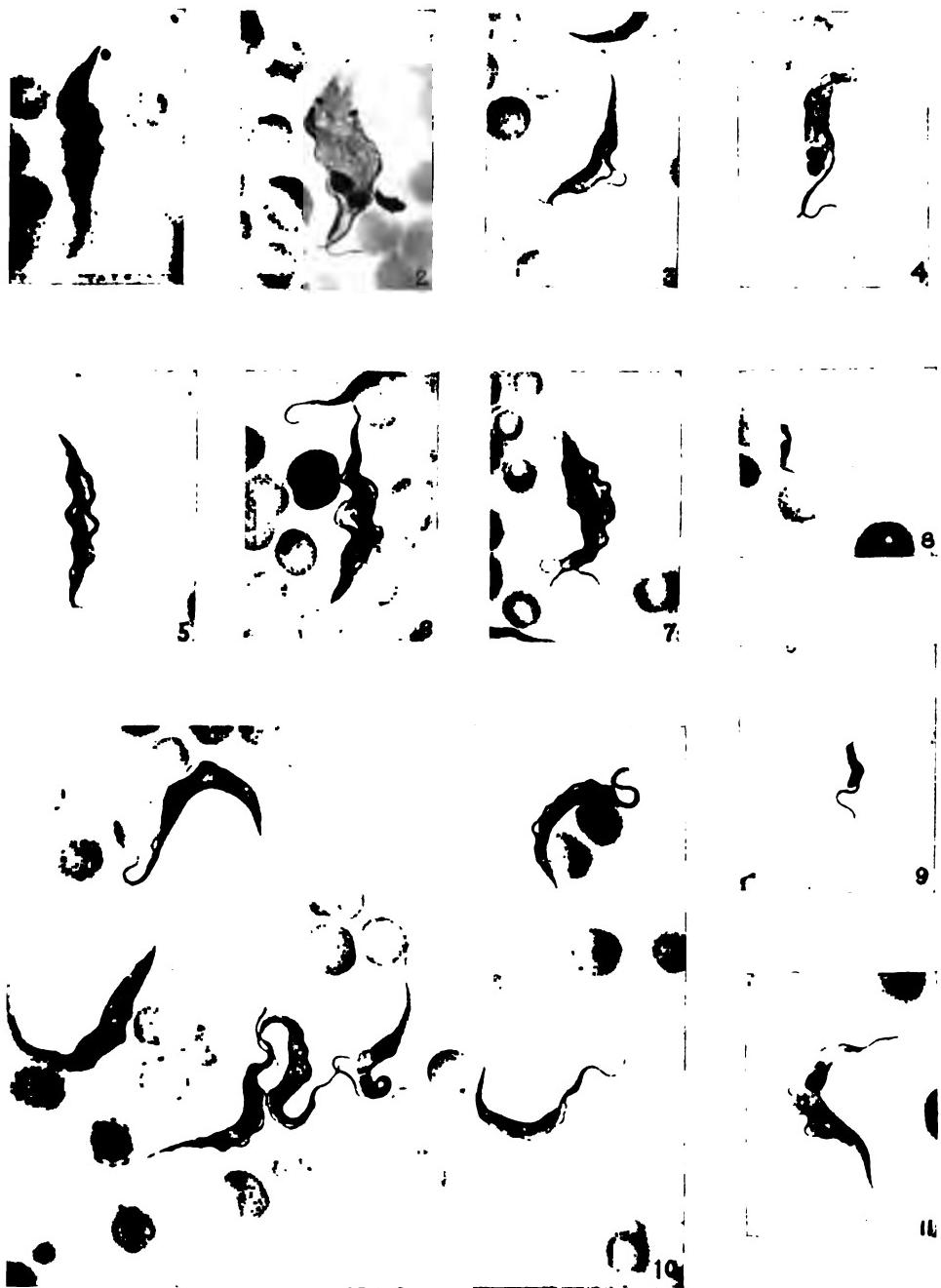
##### PLATE 67.

The photomicrographs show a magnification of 1,000 diameters, except figure 10, in which the magnification is 1,050. Figures 1, 3, 5, 7, 10, 16, 18, 21, and 22 are from smears of rat blood stained with Wright's blood stain. All the other figures are from similar preparations stained with Giemsa stain. The figures are not retouched.

FIGS. 1 to 7. Longitudinal division of *Trypanosoma lewisi*. Figures 1 and 2 show trypanosomes with the usual cytoplasmic characteristics of organisms in process of multiplication, while figures 3 to 7 represent division of older forms. Figure 3 shows division of the flagellum in a trypanosome with an elongated posterior extremity, and figure 7 multiple division of an adult trypanosome.

FIGS. 8 and 9. Small trypanosomes with the characteristics of the adult organism.

FIGS. 10 to 15. Trypanosomes with an elongated posterior extremity.



(Brown: Anomalies of Pathogenic Strain of *Trypanosoma lewisi*.)



FIG. 10. Three adult trypanosomes with posterior elongations. Compare these with the two young forms, also with elongated posterior extremities, and with the normal adult trypanosomes.

FIG. 11. A very large trypanosome with a delicately pointed posterior extremity and a highly developed undulating membrane.

PLATE 68.

FIG. 12. An extreme elongation of the posterior extremity.

FIG. 13. Posterior extremity with a bulbous tip. Compare with figure 3.

FIG. 14. Small anuclear trypanosome.

FIG. 15. A young trypanosome with posterior elongation.

FIG. 16. Short posterior extremity in an adult trypanosome.

FIGS. 17 to 19. Trypanosomes with highly developed undulating membrane. Compare with figures 11 and 13.

FIG. 20. *Trypanosoma lewisi* with a rudimentary blepharoplast. Only the dot at the root of the flagellum represents the blepharoplast. The dark line is the result of heavy staining of the flagellum crossing the body of the organism. Compare with figure 11.

FIG. 21. An ablepharoplastic trypanosome.

FIG. 22. An ablepharoplastic trypanosome with an elongated posterior extremity.

ON CERTAIN SPONTANEOUS CHICKEN TUMORS AS  
MANIFESTATIONS OF A SINGLE DISEASE.

I. SPINDLE-CELLED SARCOMATA RIFTED WITH BLOOD SINUSES.\*

BY PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 69 TO 71.

Recently three transplantable chicken tumors distinct in character have been found to have a filterable cause.<sup>1</sup> The differences between these tumors are traceable to differences in the causative agents. Each agent gives rise in normal fowls to tumors of the sort from which it was isolated by filtration, and to tumors of this sort only. For example, the agent derived from a transplantable osteochondrosarcoma gives rise to sarcomatous tumors in which cartilage and bone are laid down. Certain minor variations, it is true, do occur in each tumor strain as intercurrent phenomena. The cells of the sarcoma known in our laboratory as Chicken Tumor I are, in some chickens, of very attenuated spindle form, again oat-shaped or almost round, again interspersed with sarcomatous giant cells; and the course of the disease varies somewhat in individual fowls. But the growth is always a spindle-celled sarcoma, and its modifications are not greater than those observed in certain rat and mouse tumors propagated only by transplantation and dependent on the survival of a single race of cells. Attempts to bring about variations by injuring the filterable agent have been unsuccessful, as have attempts to make it affect epithelium.

There is good ground to suppose that other tumors of the fowl besides those already studied are caused by filterable agents. The range in structure and behavior among chicken tumors is very wide.

\* Received for publication, April 20, 1914.

<sup>1</sup> Rous, P., *Jour. Am. Med. Assn.*, 1911, lvi, 198. Rous, P., and Murphy, Jas B., *Jour. Exper. Med.*, 1914, xix, 52.

Even when composed of cells of similar origin they often exhibit, like mammalian growths, a strikingly various structure and course. Must one suppose a distinct causative agent not only for each type of neoplasm as determined morphologically, but for the almost infinite number of variations in structure and behavior of such types? The present article and the one following it deal with this point. Briefly, it has been found that two spontaneous chicken tumors recently transplanted have each given rise to neoplasms identical in composite behavior with a tumor strain already under propagation. As will be shown in the present paper, the spontaneous tumor known as Chicken Tumor 38 of our series, seems to be a manifestation of a disease-complex already reported upon and known as Chicken Tumor 18.<sup>2</sup> This latter growth is a spindle-celled sarcoma, rifted in a characteristic manner with blood sinuses and tending to metastasize to the muscles, especially in the neighborhood of joints.

#### THE SPONTANEOUS TUMORS.

The spontaneous tumor No. 38 resembled the spontaneous tumor No. 18 only in the fact that it was a growth composed of spindle cells of connective tissue origin. The fowl carrying it was a well grown but emaciated Plymouth Rock hen. It was brought to the laboratory while yet alive. The irregular tumor mass, situated in the subcutaneous tissue between the left leg and the body, measured 10 by 6 by 5 centimeters, was imperfectly encapsulated, attached to the sheath of the thigh muscles, and just beginning to involve the skin. Strands extended between the leg muscles into the drumstick. At the center of the mass was a cavity with ragged walls, containing about forty cubic centimeters of clear, straw-colored fluid. The tumor tissue was finely striated, pinkish white, rather soft, and varied with many irregular, translucent areas of colliquation. There were no metastases. Histologically the growth was composed of strands of attenuated spindle cells with much collagen, sometimes in the form of ribbons (figure 1). A few round cells were scattered here and there in the growth. There was very little resemblance to the spontaneous tumor No. 18. This latter occurred

<sup>2</sup> Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1913, xviii, 651.

in the gizzard of a brown Leghorn fowl and metastasized to several points in the skeletal muscles. Both the primary and secondary tumors consisted of a very regular spindle-celled tissue, rifted to an extraordinary degree with blood sinuses into which the growth showed a tendency to extend, with result in an intracanalicular arrangement.

The fowl carrying Chicken Tumor 38 was killed and bits of the neoplastic tissue were implanted in the breast muscle of two normal Plymouth Rock fowls, in both of which a growth slowly developed. With repeated passage the tumor's rate of growth has increased somewhat, but like No. 18 it is still much less malignant than the simple spindle-celled sarcomata, Nos. 1 and 43. It is now growing in its fourth successive series of hosts. A filterable agent causing it, distinct from the tissue cells, has been demonstrated by three methods; namely, by drying, by glycerination, and by filtration through Berkefeld cylinders impermeable to small bacteria. The findings compared with those in the case of No. 18 are, briefly, as follows:—

#### COMPARISON OF THE TUMOR STRAINS.

Both growths, whether obtained by transplantation or by the action of the filterable agent as such, are, in the gross, solid, pinkish white, unencapsulated, firm, and markedly resistant to the knife. When growing in voluntary muscle they tend to bind the fibers and limit motion, a feature not observed in the case of the simple spindle-celled sarcomata already mentioned. Both growths are composed of attenuated spindle cells arranged, often very regularly, in bundles or strands with much collagen which is usually in the form of bands or ribbons (figure 2). Giant cells are not present. The rifting with blood sinuses, which was so important a feature of the earlier generations of Chicken Tumor 18, is now only occasionally seen in this growth. Absent at first from Chicken Tumor 38, it has recently been met with in several cases (figure 3). Histologically the two growths are at present practically indistinguishable.

Chicken Tumor 18 in its earlier generations showed a notable tendency to metastasize to the skeletal muscles, especially in the neighborhood of joints. The lungs, heart, and liver were affected

sometimes, though rarely. Among nine fowls which have thus far died of Tumor 38, one had secondary tumors in lung, liver, and gizzard. In three cases there were metastases in the skeletal muscles. The simple, spindle-celled sarcomata, Nos. 1 and 43, have never shown this feature. Of the three instances referred to, one had a nodule in the wing, a second both wing and leg metastases, while in the third the involvement was widespread. A small nodule was present in the lung, larger secondary growths in the muscles of the neck, legs, and hip, and a series of coalescing masses connected the junctions of the sternal and vertebral ribs, forming what may be called a neoplastic rosary (figure 5). The whole condition closely resembled that in a fowl which died of tumors produced by the injection of a filtrate of Chicken Tumor 18.<sup>8</sup>

Tumor 18, though spontaneous in a brown Leghorn fowl, grows much better in the Plymouth Rock variety, a fact only recently determined and still to be reported upon in full. Chicken Tumor 38 likewise succeeds better in Plymouth Rocks than in brown Leghorns. But the two cases are hardly to be compared, for Tumor 38 occurred spontaneously in a Plymouth Rock fowl, and, from what is known of the laws governing transplantation, might be expected to succeed best in hosts of this sort, as indeed it does. Tumor 18 acts against the rule, growing better in hosts of an alien variety.

#### THE FILTERABLE AGENTS.

A causative agent for Chicken Tumor 18, as distinct from the cells, has been demonstrated only by filtration experiments. The dried or glycerinated tumor tissue is incapable of causing the growth. Tissue of No. 38, dried or glycerinated, gives rise to the tumor in a considerable percentage of normal fowls injected, and this within a few weeks. The Berkefeld filtrate of an extract of the growth acts almost as quickly. Filtrates derived from No. 18, on the other hand, seldom cause a tumor until several months after the injection. The differences in resistance and activity of the causative agents, as thus indicated, are the only points of dissimilarity between the tumors at present.

<sup>8</sup> Rous and Lange, *loc. cit.*

## CHICKEN TUMOR 27.

All in all, the findings give one good reason to suppose that the spontaneous chicken tumors, Nos. 18 and 38, are different manifestations of a single disease-complex. It has seemed possible that other expressions of this complex might be present among our forty-five spontaneous neoplasms of the fowl. A search shows that the growth known as No. 27 is probably such a case. The host, a brown Leghorn hen, had several large lumps in the muscles of the wings and legs which limited motion markedly, a small nodule in the gizzard, and a number of raised, sharply defined, plateau-like masses in the skin, some deeply pitted with feather follicles and one of them ulcerated. It was impossible to say which growth was primary. All consisted of a close textured, finely striated, firm, pink, sarcomatous tissue. At the time, the case appeared unique, and indeed among the spontaneous growths subsequently obtained none has given a similar picture. But among the many fowls dying of transplantation tumors of No. 18,—now in its eleventh successive series of hosts,—a single instance closely resembling that of No. 27 has been met with. The fowl, of the second transplantation generation, is mentioned in a previous article.<sup>4</sup> The discoid masses in the skin consisted, as in the case of Tumor 27, of a sharply defined, nearly homogeneous, spindle-celled, sarcomatous tissue in the looser layers of the corium, the masses in the muscle of the same sarcoma arranged for the most part in the familiar intracanalicular pattern (figure 4). Unfortunately no adequate attempt was made to transplant Tumor 27. The other spontaneous chicken tumors do not suggest, even remotely, the disease-complex of Nos. 18 and 38.

## SUMMARY.

Two spontaneous chicken tumors, unlike in several important respects, have given rise on transplantation to neoplasms of identical character. The spontaneous growth, No. 18, situated in the gizzard, was a spindle-celled sarcoma rifted with blood sinuses into which it extended, with result in what may be described as an intra-

<sup>4</sup> Rous and Lange, *loc. cit.*

canalicular pattern. The metastases, which were in the voluntary muscles, showed the same peculiar structure. Tumor 38, occurring in the subcutaneous tissue of the groin, was a solid, spindle-celled sarcoma of rather close texture, with few blood vessels. Here and there were small areas of softening, and at its center was a large degeneration cyst with ragged walls, containing a clear fluid. There were no metastases. The transplantation tumors from both growths have been characterized by slow growth, tendency to metastasize to the skeletal muscles without involvement of the lungs, and a structure which at one time is that of a very regular spindle-celled sarcoma containing many bands and ribbons of collagen, and at another that of a sarcoma rifted with blood sinuses like the spontaneous tumor No. 18. At present the two strains are practically indistinguishable in appearance and general behavior. Both are caused by filterable agents. The agent causing No. 38, unlike that causing No. 18, retains its activity in tumor tissue which has been dried or glycerinated; and in a Berkefeld filtrate it is much the more active in causing tumors. These differences can hardly be thought of as constituting a fundamental distinction between agents which, to judge from their effects, are almost undoubtedly different strains of a single disease cause.

That chicken tumors of markedly different type have different filterable agents as their cause has been proved by experiments already reported. The present findings make it probable that, within certain limits, tumors of rather various character may be dependent upon a single agent. This assumption greatly simplifies the etiological problem. But the truth of the assumption for other instances than those described in the present article can only be determined by the study and comparison in many hosts of the disease-complexes of which each spontaneous chicken tumor is to be considered as an individual expression.

EXPLANATION OF PLATES.<sup>5</sup>

## PLATE 69.

FIG. 1. Section of the spontaneous tumor No. 38. It is composed of spindle cells in strands, with abundant collagen. There is some round-celled infiltration.

FIG. 2. A solid growth of the third generation of transplants. The heavy black spots are artefacts.

## PLATE 70.

FIG. 3. Portion of a growth that resulted from the injection into a normal fowl of tumor tissue that had been dried while frozen. The rifting with blood sinuses here shown has been found in several transplantation growths as well.

FIG. 4. Spontaneous Tumor 27. Section of one of the growths in the skeletal muscles.

## PLATE 71.

FIG. 5. Secondary growths in a fowl of the first transplantation series of Tumor 38. The primary growths were situated in the pectoral muscles. They have been removed with the sternum except for a small portion of that on the left (A). There are metastases in both legs near the knee (B), in the neck muscles (C), and in the muscles within the bony trunk (D). A number of nodules coalescing into a thick cord (E) connect the junctions of the sternal and vertebral ribs.

<sup>5</sup> The microscopic sections were stained with methylene blue and eosin. The illustrations should be compared with those of Chicken Tumor 18 (Rous and Lange, *loc. cit.*).

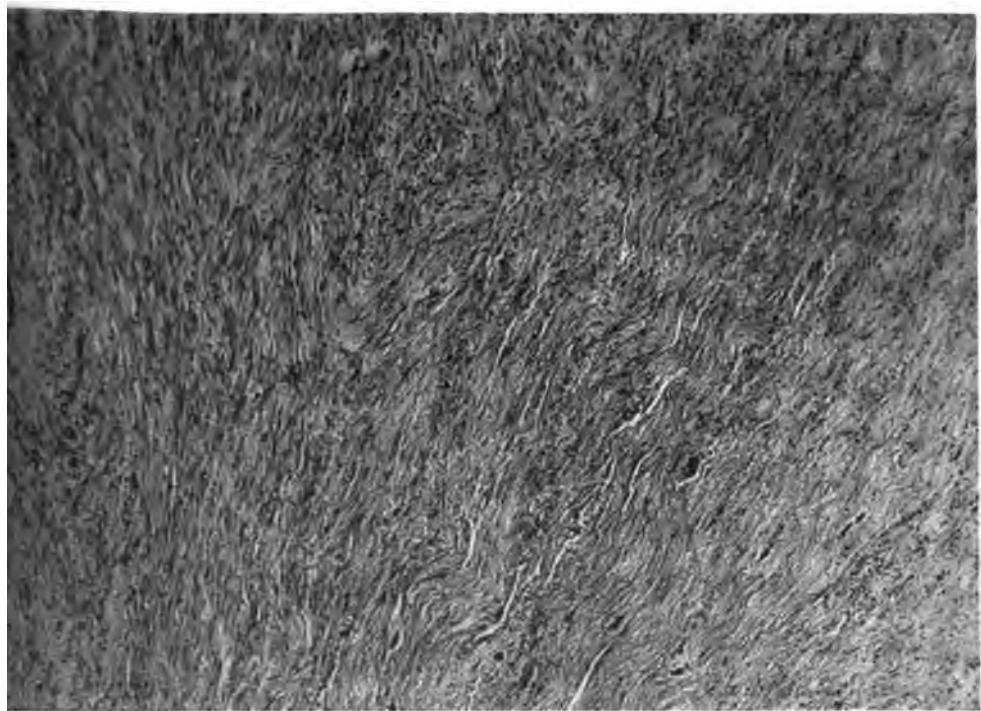


FIG. 1.

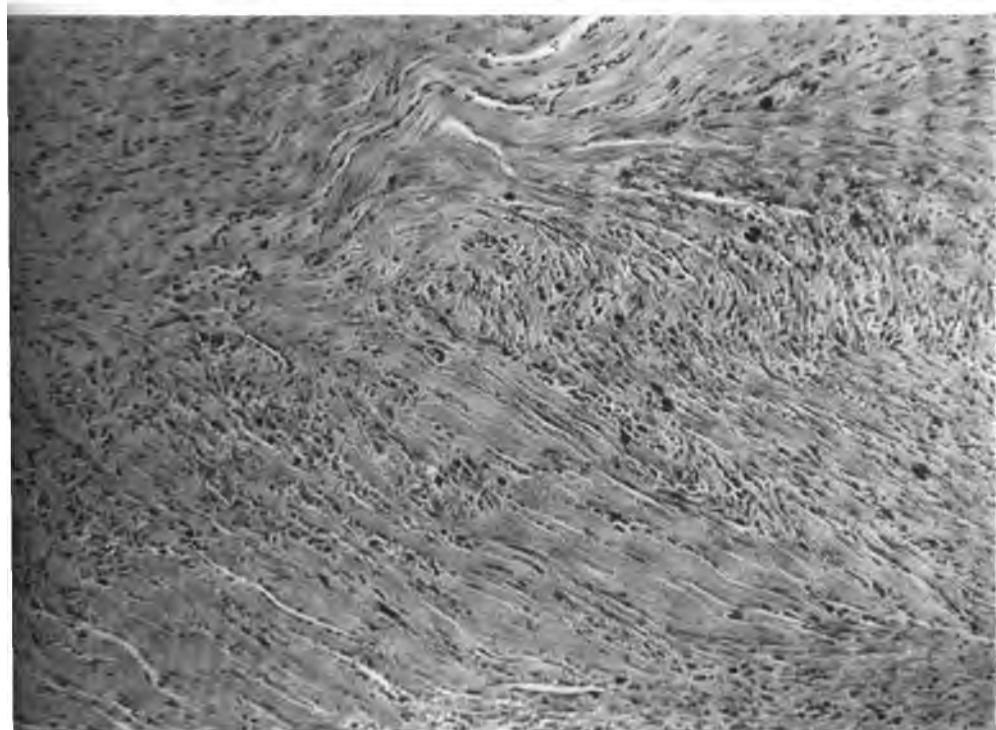


FIG. 2.

(Rous: Spontaneous Chicken Tumors.)



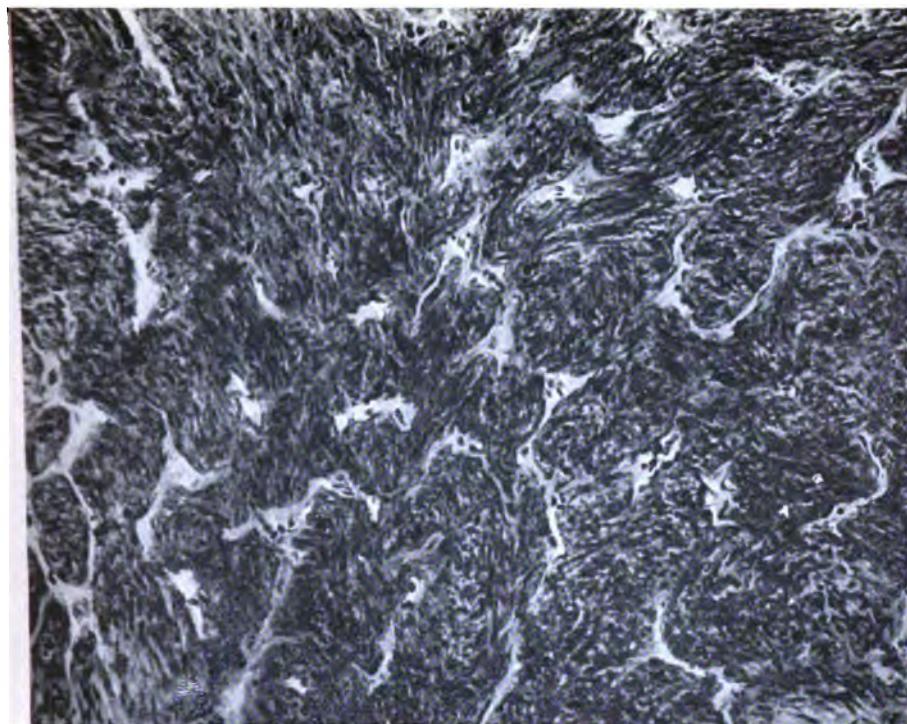


FIG. 3

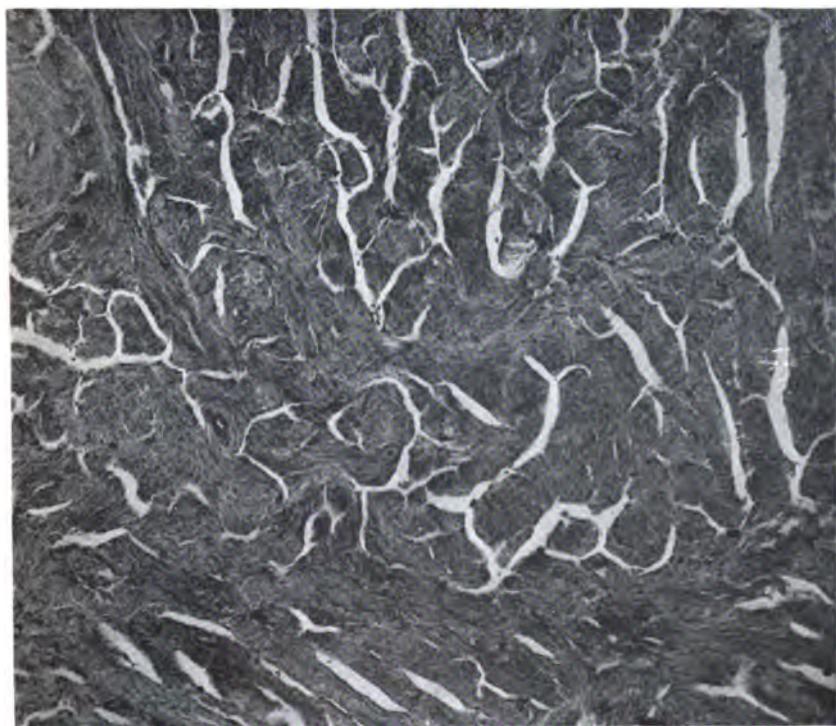
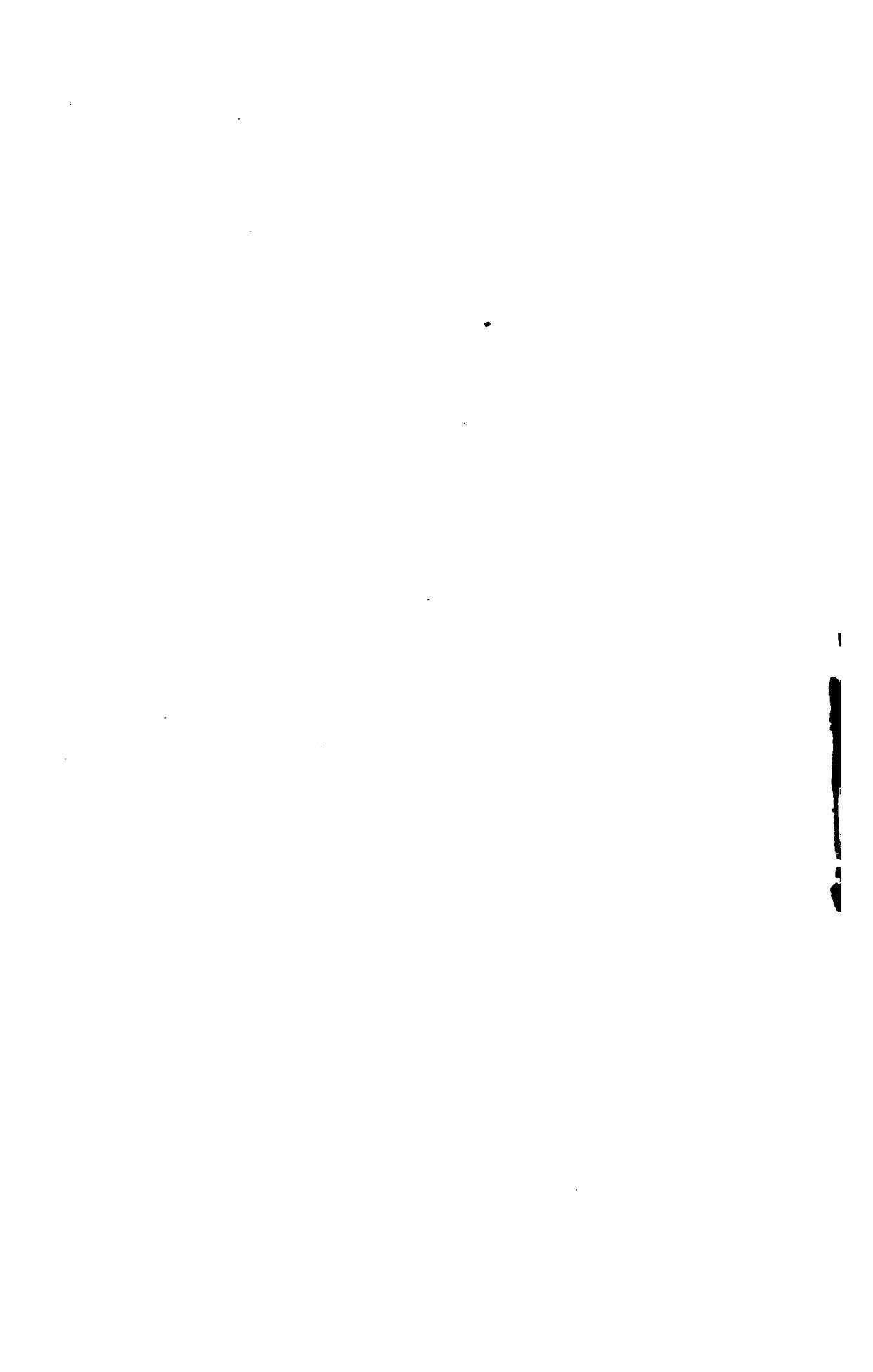


FIG. 4.

(Rous: Spontaneous Chicken Tumors.)



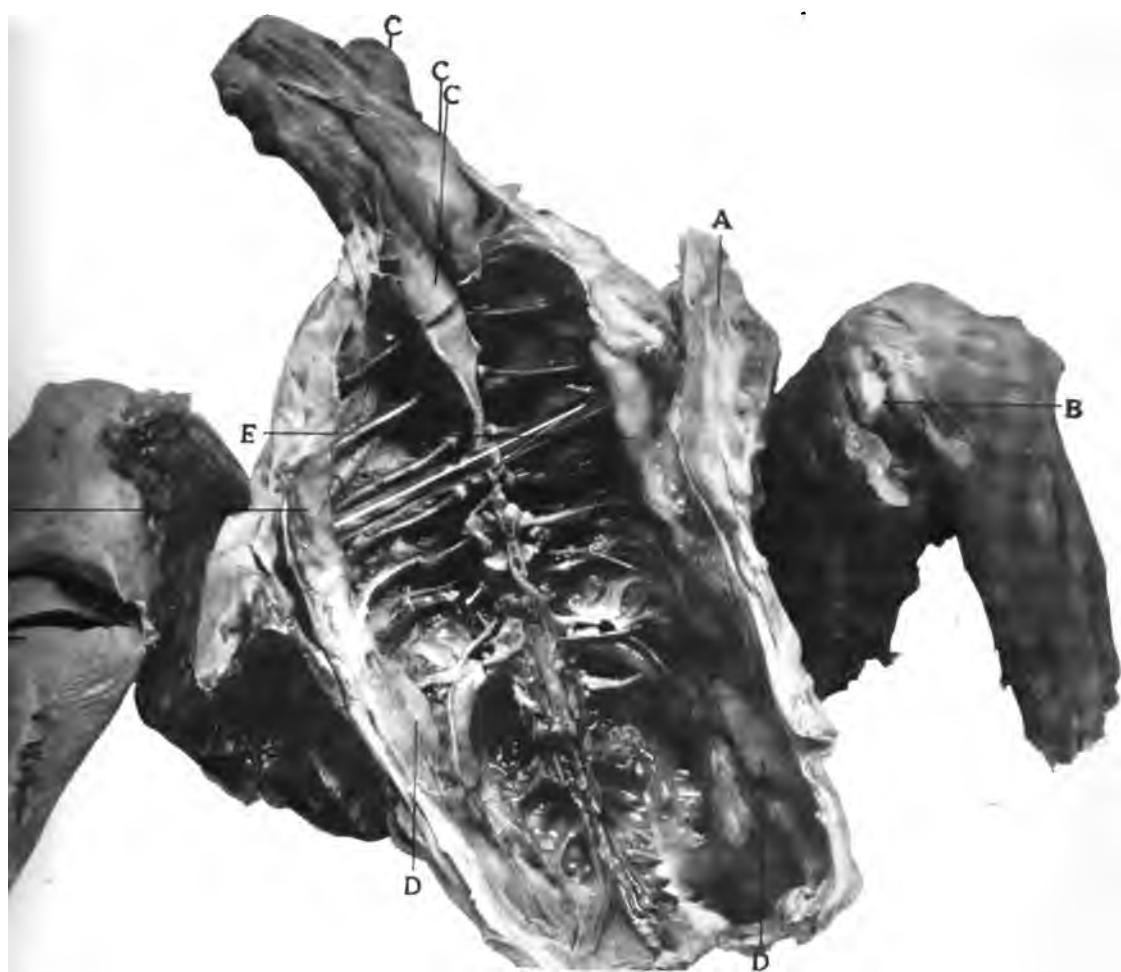


FIG. 5.

(Rous: Spontaneous Chicken Tumors.)



## ON CERTAIN SPONTANEOUS CHICKEN TUMORS AS MANIFESTATIONS OF A SINGLE DISEASE.

### II. SIMPLE SPINDLE-CELLED SARCOMATA.\*

By LINDA B. LANGE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 72 TO 74

Among the spontaneous chicken tumors recently brought to this laboratory there have been two spindle-celled sarcomata that have yielded, on transplantation, neoplasms similar respectively to two strains already under propagation. The resemblance of the growths derived from Chicken Tumor 38 to those derived from Chicken Tumor 18 is taken up in the preceding article.<sup>1</sup> They are spindle-celled sarcomata of protean character, often rifted with blood sinuses in a characteristic manner and showing a tendency to metastasize to the voluntary muscles. The subject of the present paper is Chicken Tumor 43, a simple spindle-celled sarcoma, apparently identical with Chicken Tumor 1.

The spontaneous tumor No. 38 differed considerably from the spontaneous tumor No. 18, and only after the growths had been observed in many hosts was their close similarity realized. Tumor 43, on the other hand, in its original form strikingly suggested Tumor 1, and the transplantation growths are practically identical with those of the latter. Both are produced by a filterable agent.

#### GROSS CHARACTERISTICS.

The original Chicken Tumor 43 occurred in a Plymouth Rock hen as a large nodular mass in the substance of the pectoral muscle, loosely attached to the lower end of the sternal keel, but not in-

\* Received for publication, April 20, 1914.

<sup>1</sup> Rous, P., *Jour. Exper. Med.*, 1914, xix, 570.

## RESEMBLANCE TO CHICKEN TUMOR I.

The spontaneous Chicken Tumor 43 and its transplanted growths closely resemble those of the strain known as Chicken Tumor 1.<sup>2</sup> They are of similar gross and microscopic structure, run the same course, metastasize, in general, to the same organs and have the same general action on the host. In appearance and behavior they are, indeed, indistinguishable. An etiological agent distinct from the living cells is easily demonstrated for both tumors by filtration, desiccation, and glycerination. In the absence of definite experiments upon the point it cannot be affirmed that both tumors have the same cause, yet a closer parallelism between two strains of the same disease obtained by transfer from separate instances occurring in nature could hardly be looked for. In this connection it is of interest to note that Chicken Tumor 13 (figure 5), a growth arising in the connective tissue of the foot, has some resemblance in its histology to Nos. 1 and 43, though it may not with certainty be classed with them. The attempt to propagate No. 13 was unsuccessful and nothing can be said as to its etiology.

## SUMMARY.

The forty-third spontaneous chicken tumor received at this laboratory strikingly resembles the first and has given rise on transplantation to an entirely similar series of neoplasms. Tumors of both strains are due to a filterable agent which remains active in the dried or glycerinated tissue.

EXPLANATION OF PLATES.<sup>3</sup>

## PLATE 72.

FIG. 1. Characteristic tumor filling out the left breast of the fowl. The right breast shows the emaciated state of the fowl. Metastases can be seen in the heart, lungs, and liver, as indicated by the arrows. This tumor arose from glycerinated tissue.

FIG. 2. Metastasis in the heart from a tumor of the eighth transplantation generation. There is a complete absence of a cellular reaction about the growth.

## PLATE 73.

FIG. 3. Section of the original growth showing subcutaneous spindle-celled neoplasm with scattered giant cells.

FIG. 4. Tumor of the sixth transplantation generation invading striated muscle. The muscle fibers are directly replaced by tumor cells.

## PLATE 74.

FIG. 5. Section of Chicken Tumor 13.

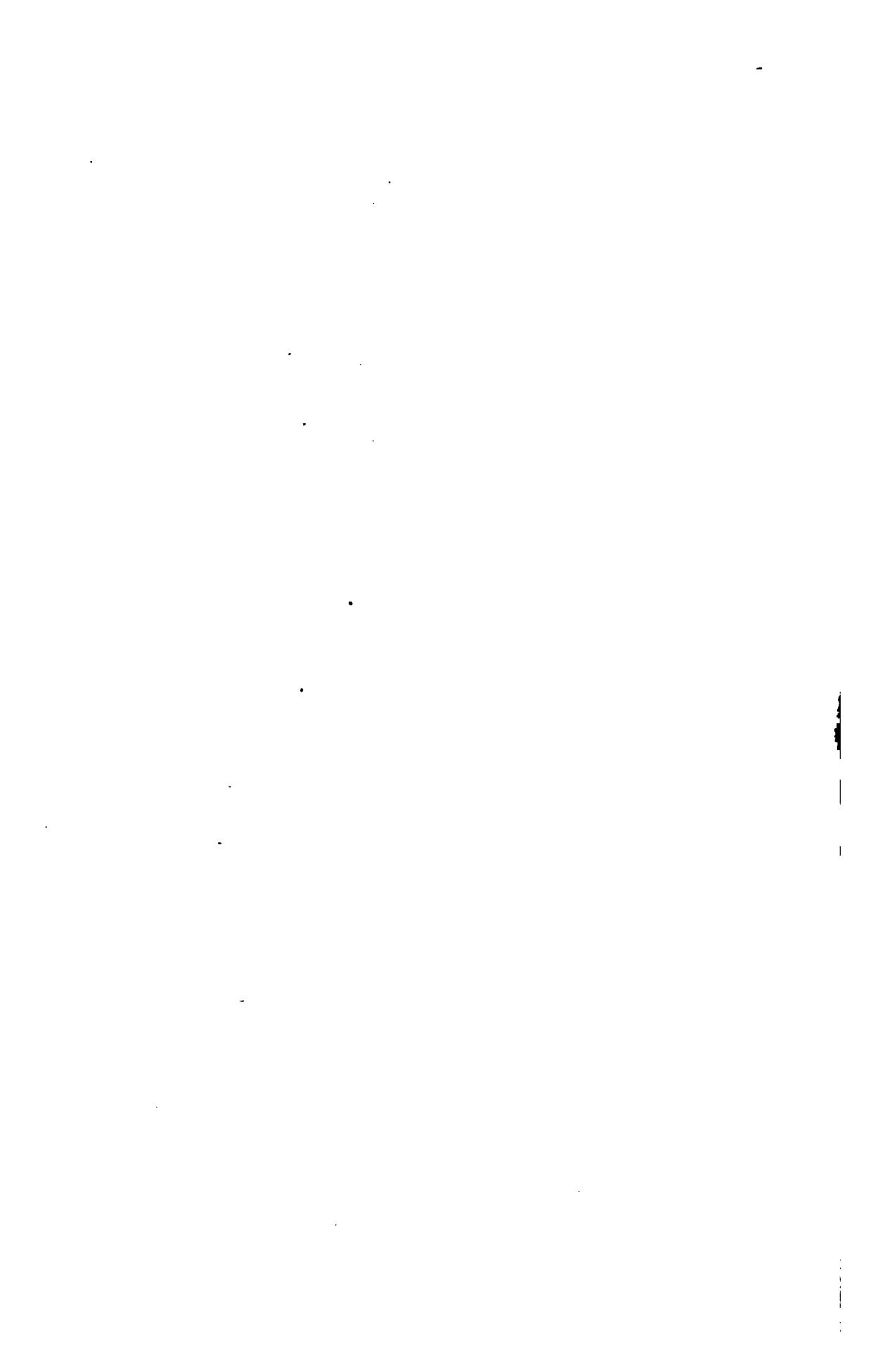
<sup>2</sup> Rous, P., *Jour. Exper. Med.*, 1910, xii, 696; 1911, xiii, 397.

<sup>3</sup> The microscopic sections were stained with methylene blue and eosin.



FIG. 1.

(Lange: Spontaneous Chicken Tumors.)



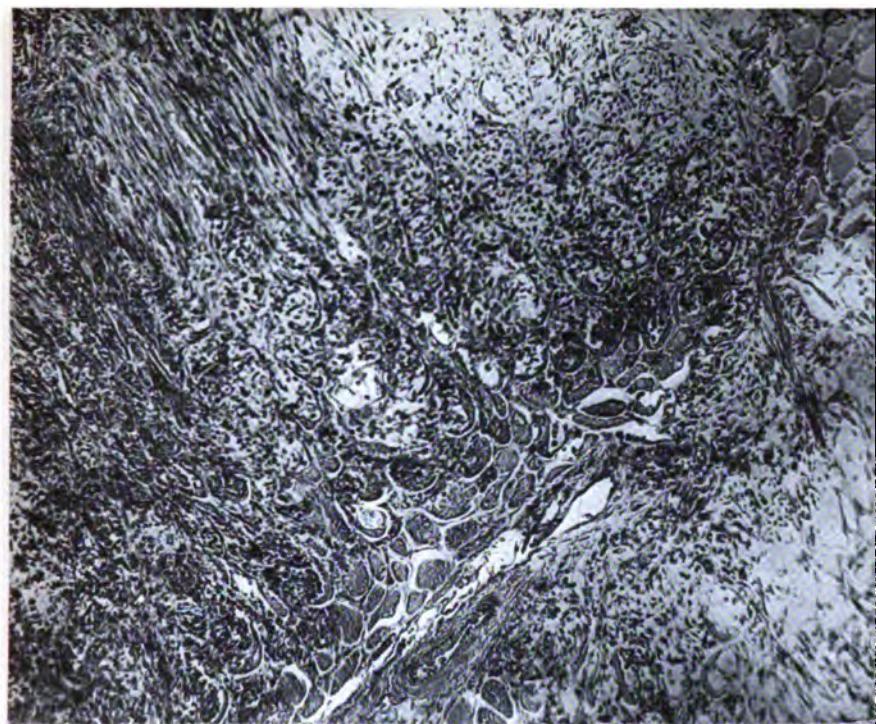


FIG. 2.

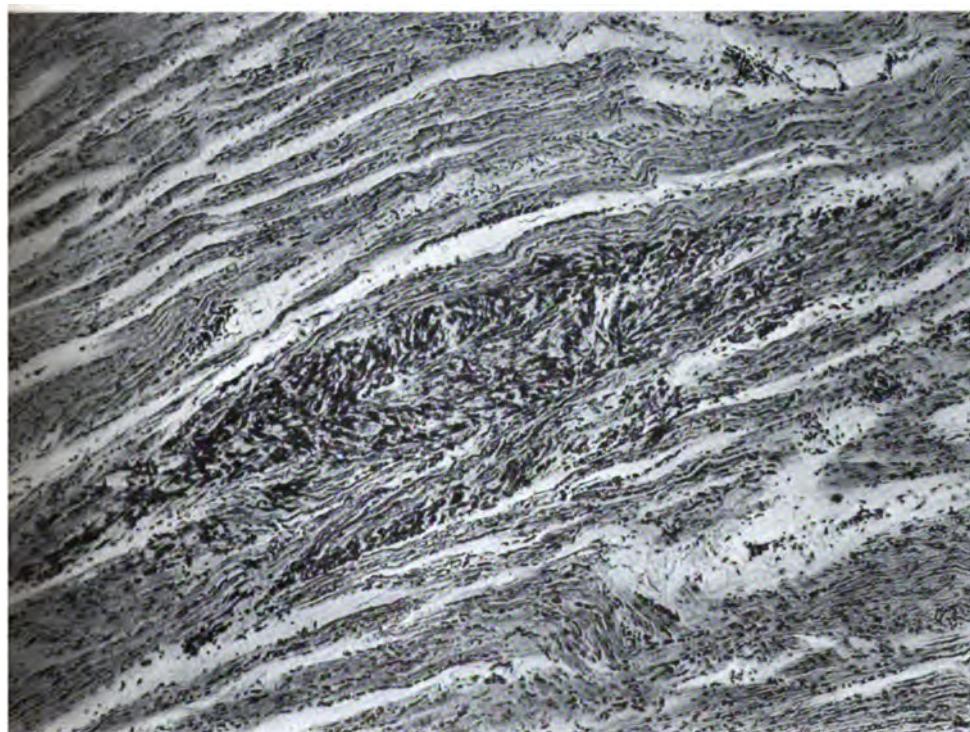


FIG. 3.

(Lange: Spontaneous Chicken Tumors.)





FIG. 4.

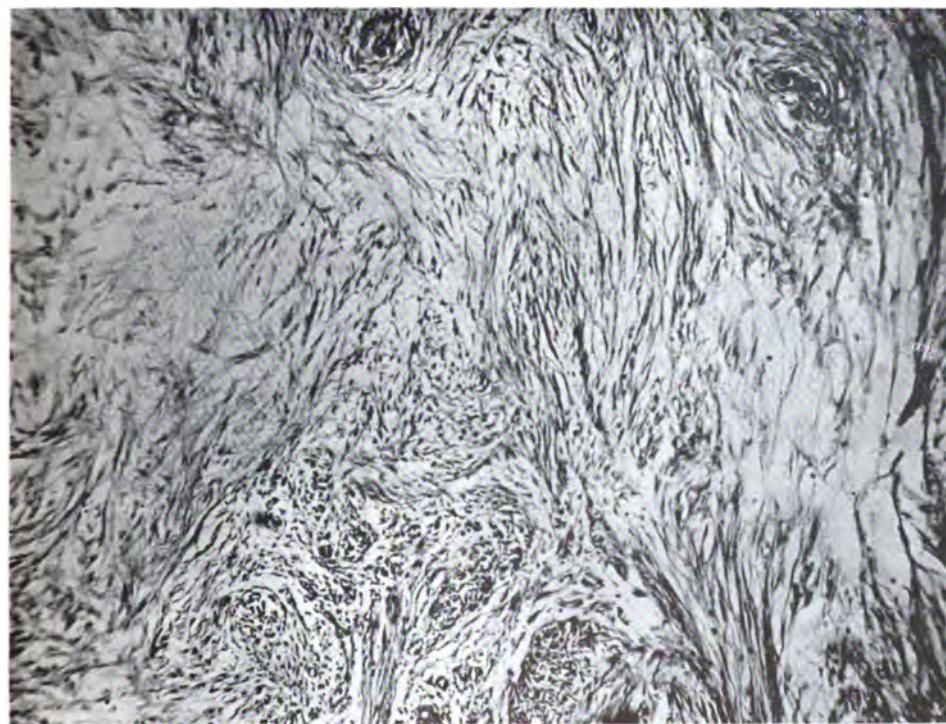
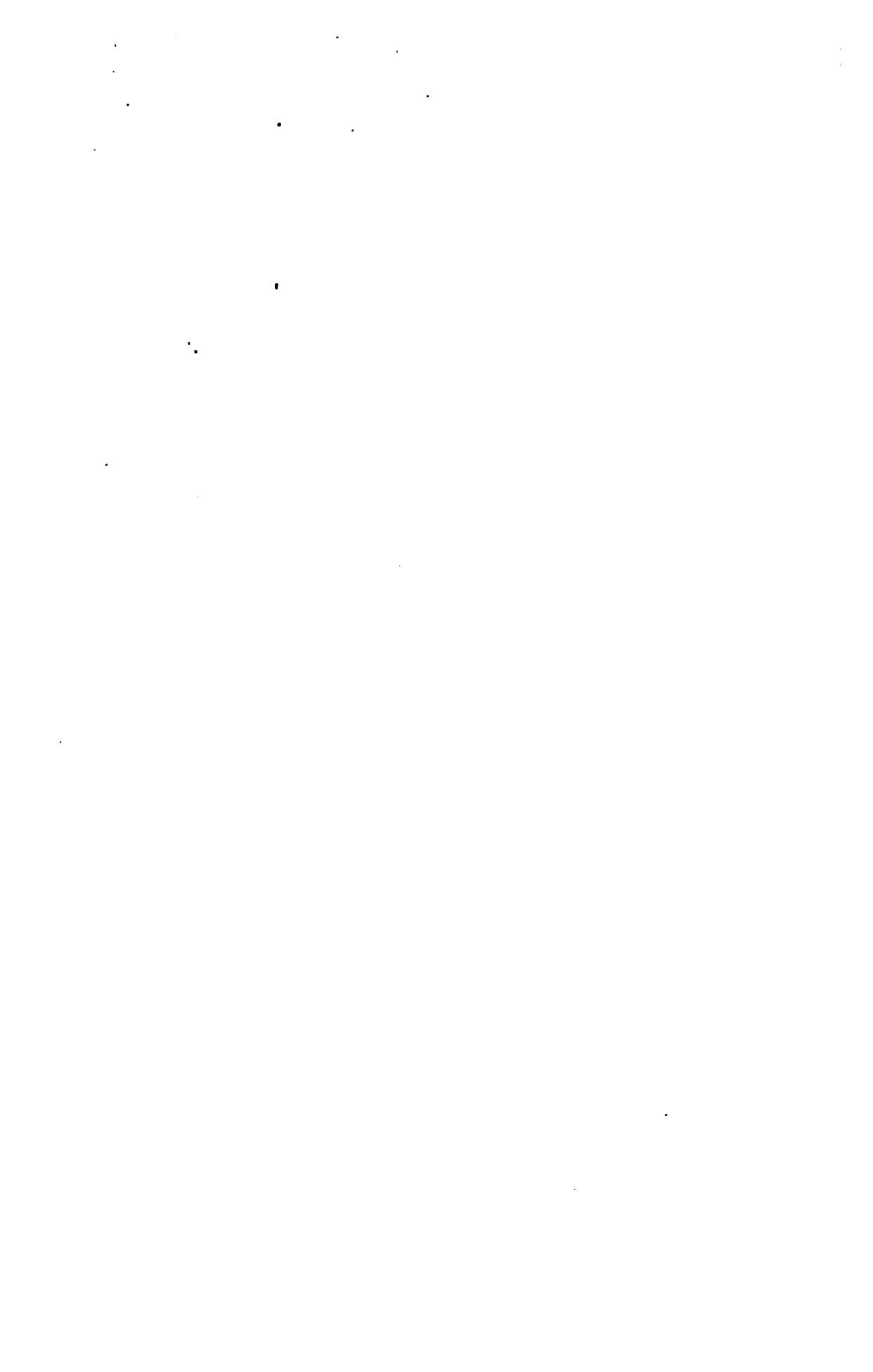


FIG. 5.

(Lange: Spontaneous Chicken Tumors.)



ROSEBANK  
HOSPITAL.

## THE RECOGNITION OF THE CHOLERA VIBRIO.\*<sup>1</sup>

By C. V. CRASTER, M.D.

(From the State of New York Health Officer's Department, Rosebank.)

Curved organisms resembling in many ways the cholera vibrio of Koch are known to occur widely in nature, chiefly as saprophytes externally, and also incidentally in one or more of the fluid secretions of the body. It is only during the prevalence of cholera epidemics that the presence of a comma-shaped organism in the intestinal contents of a person sick with an acute diarrhea may be regarded upon morphology alone as strong presumptive evidence of an infection by the specific vibrio.

### THE CHOLERA-LIKE VIBRIOS.

Saprophytic cholera-like vibrios have been found in healthy persons from infected localities, and in those suffering from diseases other than cholera, and in the waters of rivers and wells of cholera-infected districts. The morphology and cultural appearances of these vibrios at times closely approximate those of the cholera organism, differences being usually of degree only; so that we may say with the exception of the negative reaction with anticholera sera, the cholera and non-cholera vibrios appear closely related to a remarkable extent. For this reason the gross appearances, which originally were considered by Koch so distinctive as to make possible a definite diagnosis of cholera alone, have been found to be the characteristic features of a large class of bacteria, many members of which have been found to be able to sustain existence in the fluids of the body as well as to lead a saprophytic life outside.

As far as we know, the cholera-like vibrios are non-pathogenic to man, but how far it is justifiable to assume that all non-aggluti-

\* Received for publication, March 30, 1914.

<sup>1</sup> In the preparation of this article I have to acknowledge the kind assistance of Dr. Meriggio Serrati, of the Italian Royal Navy.

nating vibrios are invariably of a permanent harmless saprophytic type has been for some time a question of conjecture, especially in those instances where cholera cases have occurred in the vicinity within a recent period of time, and where infection by an attenuated variety of the cholera vibrio cannot be ruled out altogether as a possibility. It is certain that wherever cholera cases have occurred proper search has revealed the concurrent existence of the non-agglutinating vibrios.

#### CHOLERA-LIKE VIBRIOS IN CHOLERA EPIDEMICS.

Gottschlich, at Tor in Egypt in 1905-1906, isolated 48 non-agglutinating vibrios from cases of acute dysentery; and in 1911 on examining 1,160 pilgrims he found 31 cholera bacillus carriers and 23 carriers of non-agglutinating vibrios.

MacLaughlin,<sup>2</sup> at Manila in 1908, made a bacteriological examination of 36 cholera contacts, and from them isolated 27 cholera vibrios, and 46 non-agglutinating cholera-like vibrios.

The presence of non-agglutinating vibrios was particularly remarked during the months preceding the cholera epidemic in St. Petersburg in 1910, during which time cases of gastro-enteritis occurred with great frequency, and from the dejecta of some of the patients vibrios were isolated which did not agglutinate with specific anticholera sera and which had therefore to be classed as of a non-cholera nature. The etiological relation between the cholera-like organisms and the concurrent disease, although significant, was not regarded as definitely established.

#### VARIATIONS IN CHOLERA STRAINS.

It has been frequently noted that the Asiatic vibrio possesses to a marked degree the tendency of the group to undergo polymorphism, the development of atypical forms, which may occur in recently isolated strains from cholera cases and carriers, as well as in old laboratory cultures. Notable differences in morphology have been observed in cholera vibrios isolated from different cholera cases in the same epidemic.

During the epidemic in St. Petersburg in 1909-1910, Horowitz<sup>3</sup> noticed that the morphology of the cholera vibrios was not constant, and that under varying conditions the same vibrio could present such divergences from type as to become unrecognizable. The tendency to modification from type occurs also in the cultural properties which have been found to vary considerably in different

<sup>2</sup> MacLaughlin, *Philippine Hosp. Rep.*, 1912, xxvii, 381.

<sup>3</sup> Horowitz, A., *Bull. de l'Inst. Pasteur*, 1911, ix, 786.

strains, although some of these may persist more than others. The ability to liquefy gelatin, for instance, is generally present in the cholera vibrio, as it is in most of the cholera-like vibrios.<sup>4</sup> Virulence upon animals is not a constant property and may be altogether absent in recently isolated typical cultures from cholera cases.

Zlatogoroff<sup>5</sup> states that the virulence to guinea pigs of some non-agglutinating vibrios exceeds that of the cholera vibrio. The indol reaction has proved to be so uncertain that it is of little value in a final differentiation. Liefmann and Nieter<sup>6</sup> and others have shown that cholera vibrios possess hemolytic action upon blood media, as well as do the other members of this class of bacteria.

#### SERUM DIAGNOSIS.

It is by reason of these resemblances in form and culture between the cholera and cholera-like vibrios, that certain biological properties have become of importance for the purpose of diagnosis, and by means of these it is generally assumed that we have been able to recognize fundamental differences in the behavior of the typical Asiatic organism and other members of the same group.

It is well known that the presence of the specific vibrio in the body of cholera patients and of highly immunized animals causes strong activation of those tissues which bring about the formation of cellular antibodies, leading to the phenomena of agglutination, bacteriolysis, complement deviation, and serum precipitation, when the specific antigen is added to the separated immune serum. Of all pathogenic organisms the cholera vibrio is the most susceptible to the action of serum antibodies, positive agglutination taking place in extreme dilutions of the antiserum (1 to 10,000 and 1 to 40,000); and similarly with a strong bactericidal serum, bacteriolysis may be observed by Bordet's method *in vitro* in high dilutions, or *in vivo* by means of Pfeiffer's method. The reactions with the anticholera serum may be said to be specific for the cholera vibrio, and it has been considered that when we are dealing with a typical cholera culture these tests enable us to come to a definite conclusion as to whether or not we have the specific organism or a cholera-like vibrio before us. By the serum reactions, epidemic vibrios have been divided into two classes: the first includes those which agglutinate

<sup>4</sup> Craster, C. V., *Jour. Infect. Dis.*, 1913, xii, 472.

<sup>5</sup> Zlatogoroff, S. J., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1908, xlviii, 684.

<sup>6</sup> Liefmann, H., and Nieter, A., *Med. Klin.*, 1906, ii, 254.

with a specific serum in high dilution, give a positive Pfeiffer reaction, and are capable of producing in immunized animals a serum which will agglutinate in a specific manner other cholera cultures (cross agglutinations); the second class includes all the cholera-like vibrios which give a negative agglutination with anticholera sera, or agglutinate in low dilution only 1 to 10 and 1 to 50, and in which the Pfeiffer reaction and cross agglutination are generally negative. The existence of a third class presumes a condition of the cholera vibrio in which certain biological properties have become latent.

#### THE DEVELOPMENT OF AGGLUTINATION IN CHOLERA-LIKE VIBRIOS.

The virulence of cholera cultures is known to lapse with long continued cultivation, and agglutination power to become considerably modified under the same condition, unless maintained by repeated animal inoculation.

The possibility of a temporary disappearance of the agglutinating power of the cholera vibrio under unusual conditions of environment and association with other bacteria, outside the animal body, and its subsequent reestablishment by cultural and inoculation methods was first suggested by Zlatogoroff.<sup>7</sup> In the course of a study of eighteen different strains of non-agglutinating vibrios, isolated from the water of rivers and wells during a cholera epidemic in Saratow in 1908, he noted, after a month's culture upon ordinary laboratory media, a spontaneous development of agglutination with anticholera sera on the part of several of the vibrio cultures, which had previously given a negative reaction. Suspecting from this occurrence that with the remainder he might possibly have to deal with cholera vibrios that had become more or less changed by environmental conditions, he originated the following procedures for influencing the return of agglutinating properties in cultures of non-agglutinating vibrios: (1) repeated daily cultivation upon fresh alkaline beef agar with alternate incubation at 37° C. and cool storage at 16° to 18° C; (2) weekly passage through the peritoneal cavity of a series of guinea pigs.

Because of the slight virulence of the majority of the water vibrios, it became necessary to increase this property before successful passage through animals could be carried out, and this was accomplished by injecting with the vibrio cultures a certain quantity of dead typhoid bacilli, colon bacilli, or streptococci. The result of this treatment was that at the end of fifty-four generations, out of the eighteen vibrio strains which did not previously agglutinate ten became possessed of full agglutinating properties with an anticholera serum, in dilution of 1:10,000 and 1:20,000, a very high agglutinating titer. To establish further the cholera nature of his vibrios Zlatogoroff controlled his results by means of the complement fixation test of Bordet, in each case obtaining a con-

<sup>7</sup> Zlatogoroff, S. J., *loc. cit.*

firmation of the positive results with the agglutination reaction. As all these vibrio strains which eventually developed specific agglutination were isolated from the water of rivers and wells in Saratow, Zlatogoroff looked upon them as true cholera vibrios which had become weakened by a direct passage out of the agglutininogen from the bodies of the vibrios into the water, due either to the phenomenon of osmosis or to the action of the chemical constituents of the water upon the external capsule of the organism, changing in this way their biological reaction from a parasitic to a saprophytic type. To test further the theory of the loss of agglutination power of vibrios in water Zlatogoroff allowed a suspension of the cholera vibrio to remain in distilled water for seven days at room temperature. After centrifugalization and washing several times in distilled water, the agglutinating titer was found to have fallen from 1:5,000 to 1:1,000, and further treatment on these lines reduced the agglutination titer as low as 1:300. Barrenscheen,<sup>8</sup> repeating the experiments of Zlatogoroff, cultivated the cholera vibrio, which normally agglutinated in a dilution of 1:40,000, for eight days in distilled water, and found the agglutination titer reduced to 1:2,000, and after seven days more to 1:200. Both Zlatogoroff and Barrenscheen noted that when the cholera vibrio was kept for some days in distilled water there occurred a passing out of a substance (agglutininogen) from the bacterial bodies, which was shown by the appearance of an opalescence and later a flocculent precipitate in the centrifugalized culture liquid, after the addition of cholera immune serum. Horowitz<sup>9</sup> states that about 4 per cent. of vibrios isolated from actual cholera sources eventually regained agglutinating power, either spontaneously or with the use of Metchnikoff's method by cultural symbiosis with the yellow *sarcinæ*. In confirming the cholera nature of the vibrios by cross agglutinations, Horowitz found that the antisera from animals immunized against the same vibrios did not give the same reactions with different strains of cholera vibrios. At the same time some vibrios which gave a negative agglutination with an anticholera serum were able to produce in animals a serum which had a strong agglutinating reaction upon typical cholera cultures. In the case of other vibrios isolated from the stools of cholera convalescents the cross agglutination was negative at first, but later the appearance of a positive reaction with the specific serum and the Pfeiffer phenomenon proved their cholera nature. Horowitz concludes that among the cholera vibrios were certain strains presenting biological differences which evidently indicate some form of evolutionary change brought about by the reaction of the living cell to its altered environment. Carapelle,<sup>10</sup> in examining a series of sixteen vibrio cultures isolated from water in and around the city of Palermo during the cholera epidemic of 1911, in seven of which direct fecal infection of the water was evident, noted that the specific agglutination was negative or only positive in extremely low dilution of the antiserum, although the bacteriolytic reaction *in vitro* and complement deviation tests pointed to their cholera nature. Twelve cases of cholera occurring at this time in a hospital which had been carefully guarded against outside infection, and from the household water of which non-agglutinating

<sup>8</sup> Barrenscheen, H., *Centralbl. f. Bakteriol., Ite Abt., Orig.*, 1909, 1, 261.

<sup>9</sup> Horowitz, A., *loc. cit.*

<sup>10</sup> Carapelle, *Ann. d'ig. sper.*, 1912, xxii, 497.

vibrios had been isolated, caused this observer to suspect that these vibrios might be of a cholera nature. He accordingly subjected the non-agglutinating vibrios to the procedures suggested by Zlatogoroff, and by passage through a number of guinea pigs alone a high agglutinating titer was developed in all the vibrio cultures (1:4,000). The virulence was also greatly enhanced and bacteriolysis *in vitro* became much more complete. By immunizing animals with the vibrio cultures he was able to obtain antisera which agglutinated typical cholera vibrios in maximum dilution. Carapelle thus corroborates the conclusions of Zlatogoroff that non-agglutinating vibrios obtained from water and subsequently developing agglutination with anticholera sera were really true cholera vibrios which had temporarily lost this property. In order to prove his conclusions of the loss of agglutination in water he exposed suspensions of cholera vibrios in sterile water contained in Berkefeld candles to the action of running water so that the water could wash the vibrios in suspension. After twenty-two days the agglutinating power of the cholera vibrios contained in the candles had been reduced to 1:100.

In a former communication<sup>11</sup> I described the properties of a number of cholera-like vibrios which were isolated from rectal swab cultures during a cholera outbreak at Quarantine, N. Y., in 1911.<sup>12</sup> All were obtained from persons free from any disease, the majority having been isolated some weeks after cases of cholera had ceased to arrive in the port. About 15 per cent. gave a modified positive indol reaction, and the hemolytic power upon blood agar was found to be variable for each strain. At that time the morphological and cultural differences discernible between the cholera and cholera-like vibrios were in many instances so slight that little importance could be attached to them for the purpose of differential diagnosis.

In the preliminary examination for specific agglutination, although negative results were obtained in moderately high dilutions of the antiserum, there were revealed at the time considerable differences in the reactions with many of the vibrio cultures, the differences varying from a complete negative result at any dilution to one showing moderate clumping at dilutions much below that of a typical cholera culture. This was looked upon at the time as due to the presence of group affinities. The Pfeiffer reactions carried out with the cholera-like cultures were found to be negative, and a series of cross agglutination tests with the cholera vibrio and antisera obtained by immunizing rabbits with cultures of the non-agglutinating vibrio gave unsatisfactory results on account of the low titer of the antisera produced.<sup>13</sup>

Some time later when the vibrio cultures had been kept upon laboratory media for several months an examination of the agglutination power showed that three of the cultures which had formerly agglutinated only in very low dilution of the antiserum now gave a positive reaction in slightly greater dilution (1 to 100). This re-

<sup>11</sup> Craster, *Jour. Infect. Dis.*, loc. cit.

<sup>12</sup> Craster, *Jour. Am. Med. Assn.*, 1913, lxi, 2210.

<sup>13</sup> Craster, *Jour. Infect. Dis.*, loc. cit.

sult, although not high enough to be looked upon as a specific agglutination, suggested the advantage of attempting to increase this property in the cultures and in others in which agglutinations had not so developed, upon the lines pointed out by Zlatogoroff and Carapelle. For this purpose fourteen vibrio cultures, including the three agglutinating strains, were selected. Table I shows that the

TABLE I.  
*Properties of Cholera-Like Vibrios.*

No. of vibrio culture.	Gelatin liquefaction.	Fermentation.				Hemolysis.	Indol reaction.	Pathogenicity for guinea pigs.
		Saccharose.	Glucose.	Lactose.	Maltose.			
98	+	+	+	-	+	-	+	-
103	+	+	+	-	+	+	+	-
109	+	+	+	+	+	+	-	-
125	+	+	+	-	+	-	-	-
151	+	+	+	-	-	+	-	-
219	+	+	+	+	+	+	+	-
269 <sup>14</sup>	+	+	+	-	+	+	-	-
899	+	+	+	-	+	+	-	-
2464 <sup>14</sup>	+	+	+	-	+	-	-	-
3064	+	+	+	+	+	-	-	-
3832	+	+	+	-	-	+	+	-
5947	+	+	+	+	+	+	-	-
5999	+	+	+	+	+	-	-	-
6061	+	+	+	-	+	+	+	-

Gelatin liquefaction after 72 hours. Hemolysis on rabbit blood agar. Pathogenicity tested with one whole agar slant culture upon a guinea pig weighing 250 gm.

cultural properties differ only slightly; hemolysis in rabbit blood agar is generally positive, the indol reaction is positive in a few cases, and pathogenicity to guinea pigs is absent in doses of one agar slant. The Pfeiffer reaction was negative for all.

At the commencement of experimental work vibrio cultures 103, 899, and 5,999 showed a positive agglutination with specific anti-cholera serum in dilution of 1 to 100 (table II). Cultures 151, 269, and 3,832 gave a similar reaction in dilutions of 1 to 40, and cultures 98 and 219, in dilutions of 1 to 20 only. The virulence being extremely low, special measures had to be taken to insure successful animal passage. This was attained in some cases by inject-

<sup>14</sup> Pigment former.

TABLE II.

*Agglutination of Cholera-Like Vibrios with Anticholera Serum and Pfeiffer Reaction at Commencement of Experimental Work.*

No. of vibrio culture.	Normal saline.	Normal horse serum.		Anticholera serum.					Pfeiffer test.
		1:10	1:40	1:80	1:40	1:200	1:500	1:1,000	
98	-	-	-	+	-	-	-	-	-
103	-	+	-	+	+	+	-	-	-
109	-	-	-	-	-	-	-	-	-
125	-	+	-	-	-	-	-	-	-
151	-	+	-	+	-	-	-	-	-
219	-	-	-	+	-	-	-	-	-
269	-	+	-	+	+	-	-	-	-
899	-	-	-	+	+	+	-	-	-
2464	-	-	-	-	-	-	-	-	-
3064	-	-	-	-	-	-	-	-	-
3832	-	+	-	+	+	-	-	-	-
5947	-	+	-	-	-	-	-	-	-
5999	-	-	-	+	+	+	-	-	-
6061	-	-	-	-	-	-	-	-	-

Nos. 103, 899, and 5,999 developed agglutination in 1:100 dilution of anti-serum, in laboratory cultivation after isolation.

ing two or more agar slant cultures emulsified in normal saline at one time into the peritoneal cavity of a guinea pig weighing 250 grams. When this method failed cultures of dead typhoid bacilli or *Bacillus coli* were injected at the same time.

After serial passage through five guinea pigs, and daily cultivation upon fresh alkaline agar for seventy generations, considerable increase in the agglutinating power was observed to have taken place (table III). Of the three vibrio cultures formerly agglutinating in serum dilution of 1 to 100, Nos. 899 and 5,999 showed positive results in serum dilution 1 to 2,000, and No. 103 in a dilution of 1 to 1,000.

Vibrios 269 and 3,832 increased in agglutination from dilutions of 1 to 40 to 1 to 100; and cultures 219 and 98 from 1 to 20 to 1 to 400 and 1 to 100, respectively. The response of the agglutinating power to intensive cultivation and animal passage was progressive, although the limit of agglutination was reached in some instances by the second or third animal passage, further inoculation producing no increase in this property.

TABLE III.

*Agglutination of Cholera-Like Vibrios with Anticholera Serum and Pfeiffer and Bordet Reactions after Intensive Cultivation and Passage through Guinea Pigs.*

No. of vibrio culture.	Normal horse serum.		Anticholera serum.						Pfeiffer test.	Bordet test.
	1:10	1:40	1:20	1:40	1:200	1:400	1:1,000	1:2,000		
98	-	-	+	+	+	-	-	-	-	-
103	+	-	+	+	+	+	+	-	-	+
109	-	-	-	-	-	-	-	-	-	-
125	+	-	-	-	-	-	-	-	-	-
151	+	-	+	+	-	-	-	-	-	-
219	-	-	+	+	+	+	-	-	-	+
269	+	-	+	+	+	-	-	-	-	-
899	-	-	+	+	+	+	+	+	-	+
2464	-	-	-	-	-	-	-	-	-	-
3064	-	-	-	-	-	-	-	-	-	-
3832	+	-	+	+	+	-	-	-	-	-
5947	+	-	-	-	-	-	-	-	-	-
5999	-	-	+	+	+	+	+	+	-	+
6061	-	-	-	-	-	-	-	-	-	-

The virulence upon animals had not been increased to any great extent by these methods. The most virulent culture, No. 5,999, became pathogenic to a guinea pig weighing 250 grams in doses of one platinum loop of a twenty-four hour agar-grown culture, and the others required larger doses than this to produce death of a similar sized guinea pig in twenty-four hours. To this absence of high virulence must be ascribed the failure to give a positive Pfeiffer reaction which was negative for all the vibrio cultures at the end of the period of special treatment.

For this reason the effect of an anticholera bacteriolytic serum upon the agglutinating vibrio cultures was tested *in vitro* by the method of Bordet.<sup>15</sup> The specific bacteriolytic serum was diluted so that when added to a mixture of the bacterial emulsion and fresh guinea pig serum to provide alexin (complement), dilutions of the specific serum of 1 to 200, 1 to 500, and 1 to 1,000 were obtained. Controls were made with the same quantities of the bacterial emulsion and alexin, but without the specific serum.

Hanging drop preparations of these mixtures were made, and they were incubated at 37° C. and examined in two to three hours.

<sup>15</sup> Bordet, J., *Ann. de l'Inst. Pasteur*, 1895, ix, 462.

A positive result showed complete lysis of the vibrios with the immune serum; the controls showed actively motile organisms. If the results after this time were incomplete, a further examination was made in eighteen to twenty-four hours at 37° C.

By this method positive bacteriolysis was observed to occur with cultures 103, 219, 899, and 5,999 (table III) in serum dilution of 1 to 1,000. The remaining vibrio cultures gave negative results even in low dilutions of the serum (1 to 200). Control hanging drop tests with known cholera vibrio cultures were carried out at the same time.

If we consider the results obtained by these methods, it will be seen that certain vibrio cultures which formerly gave so slight an agglutination as to be considered negative, later reacted in a specific manner with an anticholera serum. Similarly a strong bactericidal serum exerts upon them a bacteriolytic power seen only in the case of true cholera vibrios. If it cannot be said definitely that these vibrio cultures are really cholera, we are at least justified in strongly suspecting their cholera nature until other means of proving their true character are found.

Upon this hypothesis it may be agreed that the cholera vibrio in the process of attenuation in the intestinal contents at times loses simultaneously with its ability to produce the disease the power of responding to circulating agglutinins. The loss of agglutination may be due to the production by the bacterial cell of a defensive ferment of an anti-agglutinating nature, probably a necessary function for continued existence in hostile body fluids. Upon removal to an environment away from these serum antibodies the necessity for the formation of defensive substances ceases to exist, and agglutination tendencies slowly return.

Experience has shown that the exact value to be placed upon tests depending for their accuracy upon the possession of biological properties rests upon the appreciation of the margin of error which must be allowed for in their proper manipulation. It is well understood that biological properties are not always constantly manifest and are subject to variation in the same bacterial strain,—such changes being dependent upon conditions of environment, such as cultural media and the presence of other bacteria. Changes of this

character are well exemplified in the modifications observed as taking place in the agglutinating property of *Bacillus typhosus*, an organism which is peculiarly susceptible to the action of agglutinating sera. Many observers have noted that some varieties of this bacillus after isolation from typhoid stools or from infected water have shown at first little or no tendency to agglutinate with an anti-typhoid serum, but that further cultivation upon laboratory media brings about strong agglutination properties. *Bacillus typhosus* loses its agglutinating property rapidly in water, but it may be restored by cultivation in suitable media. Gay and Claypole<sup>16</sup> have noted variations in the agglutinability of freshly isolated typhoid bacilli, especially the frequent failure of typhoid blood cultures to clump with the antiserum from an animal immunized by means of agar-grown typhoid cultures. A peculiar deviation of agglutination was induced by growing the agar-cultivated organism upon blood agar for two or three generations. These cultures would then agglutinate with the serum from an animal immunized against blood media cultures but not against that of an animal immunized against agar-grown cultures. It is clear then that in the case of *Bacillus typhosus* the property of agglutination with a specific serum may be absent under natural conditions in the stools of typhoid patients, or, if present, it may be modified by growth in different media.

The agglutination property of the cholera vibrio has also been found to become considerably modified or lost under experimental conditions when cultivated upon a medium composed of bouillon and anticholera serum,<sup>17</sup> and the same result may be brought about by the use of earth and water media.<sup>18</sup> Conversely non-agglutinating vibrios if subjected to cultural symbiosis with sarcinæ may develop at times specific agglutination with an anticholera serum.<sup>19</sup>

Great variation in the agglutination of cholera cultures was observed by Pottevin<sup>20</sup> in a study of 127 vibrio cultures isolated at Tor in Egypt in 1912-1913. The agglutination power of many of the

<sup>16</sup> Gay, F. P., and Claypole, E. J., *Jour. Am. Med. Assn.*, 1913, ix, 1141.

<sup>17</sup> Ransom and Kitashima, cited by Zlatogoroff, S. J., *loc. cit.*, p. 694.

<sup>18</sup> Puntoni, V., *Policlinico, sez. med.*, 1913, xx, 385.

<sup>19</sup> Puntoni, V., *Gior. d. r. soc. ital. d'ig.*, 1913, xxxv, 289.

<sup>20</sup> Pottevin, *Bulletin de l'office internationale d'hygiène publique*, 1913, v, 1152.

cultures was seen to disappear or become latent in cultivation, and non-agglutinating vibrios at times apparently spontaneously developed specific agglutination some time after isolation. This observer further states that agglutinating and non-agglutinating vibrios could exist at the same time in one patient.

#### SUMMARY.

Cholera-like non-agglutinating vibrios are invariably found in the intestinal contents of healthy persons, and frequently in the water of wells and rivers, during epidemics of cholera. Although many of these saprophytic vibrios are indistinguishable in morphology and cultural properties from the cholera vibrio, the negative reaction with an anticholera serum has readily differentiated them from the Asiatic vibrio. The biological polymorphism of the cholera vibrio has been suggested by the development of agglutination, by special methods of culture, in cholera-like vibrios. Confirmatory Pfeiffer reactions have not been obtained, as a rule, in these instances, probably because of the low virulence of the vibrio culture, although positive bacteriolysis *in vitro* (Bordet's test) was observed in some, and in others positive complement fixation and cross agglutination indicated the cholera nature of the vibrios in question.

Although it cannot as yet be definitely proven, we are justified in suspecting that cholera-like vibrios which eventually develop agglutination properties are of a true cholera nature. It is probable that the production of agglutination antibodies in the serum brings about the development by the bacterial cell of defensive anti-agglutinins, resulting in the disappearance of agglutinating power. In the case of the water vibrios, changed physical conditions could bring about a similar alteration in biological properties.

It may be said that the absence of agglutination in a vibrio isolated from a suspected source does not define conclusively its non-cholera nature. In all probability among a number of cholera-like vibrios isolated from suspected sources a certain percentage will eventually be found to develop agglutination either during laboratory cultivation or by means of animal passage, and until subjected to a procedure that will induce the return of agglutination no vibrio can be regarded with assurance as of a truly saprophytic variety.

## THE CULTIVATION OF HUMAN TISSUE IN VITRO.\*<sup>1</sup>

By JOSEPH R. LOSEE, M.D., AND ALBERT H. EBELING.

(From the Laboratories of the New York Lying-In Hospital and of The Rockefeller Institute for Medical Research.)

PLATES 75 TO 77.

The present investigations were undertaken to ascertain whether human connective tissue taken from a fresh cadaver could be kept in a condition of permanent life outside of the organism. We have applied to human tissues the method by which Carrel was able to keep animal connective tissue alive *in vitro* for more than two years.<sup>2</sup>

The first attempt to cultivate human tissues *in vitro* was made in 1911 by Carrel and Burrows.<sup>3</sup> Small fragments of human malignant tumors were placed in human plasma and incubated. In a few cases the fragments were surrounded after a few days by many cells; but generally liquefaction of the medium occurred and no growth was observed. In other experiments undertaken later by Carrel on the growth of normal tissues the same phenomenon was observed. Fragments of thyroid gland and fragments of connective tissue from adult individuals or from fetuses were inoculated into culture media which contained or did not contain tissue extract. The liquefaction was observed in this case in the same manner as in the case of the malignant tumors, and with the exception of a few

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<sup>1</sup> These investigations were carried on in March, 1913, in the research laboratory of the New York Lying-In Hospital. They were rendered possible by the coöperation of Dr. Markoe, of the attending surgeons of the Lying-In Hospital, and of Dr. Carrel. The Directors of the Hospital provided a specially equipped laboratory for the work, under the supervision of Dr. Markoe and Dr. Carrel, thus enabling us to carry out the study of the tissues of human cadavers under excellent conditions.

<sup>2</sup> Carrel, A., *Jour. Exper. Med.*, 1913, xviii, 287.

<sup>3</sup> Carrel, A., and Burrows, M. T., *idem*, 1911, xiii, 387.

cultures no real growth was observed. In 1913 Maccabruni<sup>4</sup> cultivated in plasma the tissues of the uterus and ovary of human fetuses and observed around the tissue after 8 or 9 days the presence of many cells and also of karyokinetic figures. The result of these experiments showed that human tissue almost always produced liquefaction of the plasma, and that this probably prevented its growth. We, therefore, attempted to develop a technique which would permit us to keep human tissue in a plasmatic medium without the occurrence of liquefaction.

At first we attempted to obtain a medium which would not liquefy under the influence of the tissue. The first medium tried was composed of human plasma and extract of human tissue taken from fresh cadavers. This, however, proved unsuitable, as in twenty-four hours liquefaction occurred about the fragments of tissue. Very little growth was observed, but when it took place the cells were seen to be scattered and the new growth to be very thin. A thin film containing many cells adhered to the cover-glass. When attempts were made to extirpate the film and to transfer it to fresh medium it generally became folded up and after one or two passages no further growth occurred. Many modifications of the medium were tried, in order to overcome these difficulties. Human plasma was diluted with agar, serum, and egg albumen. The addition of agar and egg albumen to the plasma yielded a firmer medium. No liquefaction took place, but on the other hand no real growth occurred. When the plasma was diluted with serum complete liquefaction rapidly followed. The addition of a small amount of acid or alkali to the plasma did not prevent this liquefaction. Finally after many attempts had been made it was found that by diluting the plasma with equal parts of Ringer solution a medium could be obtained which would not liquefy in less than 24 hours, and often not in 48 and 72 hours. Usually 18 hours after the medium had been inoculated with human tissue, growth appeared and increased progressively. After a period of from 24 to 96 hours the fragments of tissue were transferred to a fresh medium in which the growth continued. The medium was again modified by the addition of a small quantity of diluted extract of human tissue, after which the

<sup>4</sup> Maccabruni, F., *Ann. d. ostet. e ginecol.*, 1914, xxxvi, 57.

growth became very active. Finally at the end of 1913 and during the first months of 1914 it became possible to obtain large growths of human connective tissue. The tissue could be transferred from medium to medium; the new cells could be isolated from a primitive fragment and cultivated in a new medium; the mass of newly formed tissue rapidly increased in the same way that was observed by Carrel to occur in the case of the strain of connective tissue cells taken from the chick embryo, which are still proliferating at The Rockefeller Institute. Nevertheless, the technique which we have developed is still far from perfect. Liquefaction of the medium occurs as soon as the tissue extract becomes too concentrated. Besides, it appears that the plasma taken from many different individuals varies widely, the plasma of certain persons liquefying much more rapidly than that of others. The value of the extracts varies also, the result being that the medium is inconstant; and for this reason the rate of growth of fragments of tissue shows marked fluctuations in the course of a few weeks. The medium will probably be still further modified; nevertheless we shall describe here the technique that has rendered possible the cultivation of human tissue *in vitro*.

#### TECHNIQUE.

*Plasma.*—Blood is aspirated from the median basilic vein of adults under aseptic conditions with a dry, chilled, 10 c.c. glass syringe (gauge of needle, 19). The blood is immediately transferred to cold paraffined glass tubes and centrifuged in ice at high speed (3,000 revolutions) for 5 minutes. The plasma is at once pipetted off with a paraffined pipette into another cold paraffined tube, which was kept on ice ready for use. Several paraffined pipettes are prepared with bulbs to be kept in readiness, because it has been found that although the plasma remains liquid for some time in the paraffined tube it coagulates in the pipette used for making the cultures in from two to ten minutes. It is usually necessary to use a fresh pipette after changing two or three cultures.

*Extract.*—The extract is prepared from tissues taken from adult or fetal cadavers, and cut into small pieces. Equal parts of Ringer solution are added, the whole is placed in cold storage for 48 hours, after which it is centrifuged and the supernatant fluid pipetted off. This fluid may then be kept for some time in cold storage.

*Making of Cultures.*—Pieces of the various fetal tissues taken from fresh fetal cadavers are placed in Ringer solution or in serum and cut to the proper size with a cataract knife. The plasma is first spread on the cover-glass, and equal parts of Ringer solution are added and thoroughly mixed with it. The object of this

procedure is to obtain a clot which can adhere firmly to the cover-glass, because it was found that when the plasma was diluted with the Ringer solution first coagulation took place too rapidly, thereby preventing a thorough embedding of the tissue. On the other hand, when the Ringer solution is spread first and the plasma added afterwards the clot does not adhere firmly, often contracting to such an extent as to become separated from the cover-glass, thereby destroying the culture. Then the tissue is put into the medium and the extract immediately added. The medium usually coagulates about the piece within a few seconds. A reasonable amount of speed and strict aseptic precautions are observed throughout. The preparations are next placed over a concave slide hermetically sealed with paraffin and incubated at 38° C., and the tissues allowed to grow. When a fresh amount of growth has taken place after a period varying from 24 to 96 hours, the tissues are removed from their medium, washed, and transferred to a fresh medium. The time of the transfer depends upon the condition of the medium and the activity of the growth. Observations are taken at intervals of 12, 24, 48, and even 96 hours. Observations are likewise made of cultures fixed and stained with hematoxylin and with Giemsa stain.

#### RESULTS.

With the technique described above, it became possible to keep a strain of connective tissue cells, derived from a piece of skin which was obtained from a fresh four months old fetal cadaver, in a condition of active life *in vitro* for more than two months.

When the fragments of fetal heart tissue, obtained from fresh cadavers, were introduced into a medium composed of equal parts of human plasma and Ringer solution, and incubated at 38° C. for 16 hours, microscopic examination of the culture showed the fragments as sharply outlined masses in a slightly opalescent medium with no evidences of cell proliferation. In from 18 to 24 hours cell proliferation manifested itself, in some cases by the appearance of many cells spreading out or budding from the original piece; in other cases by the presence of only a few scattered cells.

The rate and extent of growth varied and probably depended on the vitality of the tissue, the constituents of the medium, and the changes that developed in it. Where there was a tendency for the proliferating cells to grow in one or two planes the growth was greater and more rapid than where the cells appeared to grow in several planes. In the latter case the growth was also more dense.

The phenomenon of growth was accompanied by a progressive and slow liquefaction of the medium around the primitive fragment

and in the zone of cell proliferation. Sometimes liquefaction around the entire fragment took place within 24 hours after the cultures were made, although little, if any, growth was apparent. At times the medium retracted from the piece in certain areas and the vacuoles in the medium thus produced contained fluid in which detached cells and débris were observed. The liquefaction did not indicate necrosis of the piece of tissue in all instances. Pieces around which the medium liquefied completely in 24 hours have been transplanted into fresh medium, in which cell proliferation took place in from 16 to 24 hours. There was no evidence of rapid liquefaction.

When liquefaction was apparently due to cell proliferation, it started from the periphery of the fragment and was progressive. On the other hand, when apparently due to substances in the medium it was general and affected the entire mass of the medium. Liquefaction of the entire medium took place when extract from fetal tissues was used in primitive cultures. Primitive fragments grew well without the addition of extract to the medium for one or two passages, but usually after the second passage it was necessary to add extract in order to stimulate growth. The time of the passages into fresh medium was governed by the condition of the culture. Where the medium showed liquefaction around the primitive piece in 24 hours, a change was made. If the rate of growth was rapid, the culture was changed after 48 hours. Actively growing cultures which were not changed until after 72 hours grew less well. In cases where the time of passage was deferred for 96 hours, subsequent passages showed a decided retardation in the rate and extent of growth of the cells and the cellular characters were altered. Ameboid cells occurred and rapid liquefaction of the entire medium often took place; and although passages into fresh medium were made every 24 or 48 hours the cultures did not recover.

In the history of one culture, which is given in table I, it will be noticed that 24 hours after the 33d passage excellent growth was recorded; 96 hours later the culture was changed, and in 24 hours observation showed retarded cell proliferation with subsequent liquefaction. After the 34th passage, that is, 68 days, there was no further growth and death of the culture gradually resulted. In the

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 121-1	Oct. 21	Culture of skin from a 4 months' fetal cadaver cultivated in 1 drop of plasma and 1 drop of Ringer solution	Oct. 22. Medium in good condition; no growth. Oct. 23. Medium in good condition; growing. Oct. 24. Medium slightly liquefied; growth continuing. Oct. 25. Medium liquefied; no growth.
	Oct. 24	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 drop of extract	Oct. 26. Medium in good condition; growing. Oct. 27. Medium partially liquefied; growing.
	Oct. 25	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 small drop of extract	Oct. 28. Medium slightly liquefied; growing. Oct. 29. Medium liquefied; growing.
	Oct. 27	Washed in Ringer solution for $\frac{1}{2}$ minute and cultivated in the same medium	Oct. 30. Medium completely liquefied; some growth.
	Oct. 29	Washed in Ringer solution for $\frac{1}{2}$ minute and cultivated in 2 drops of serum, 1 drop of plasma, and 1 small drop of extract	Oct. 31. Medium in good condition; excellent growth.
	Oct. 30	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 small drop of extract	Nov. 1. Excellent growth.
	Nov. 1	Divided into 2 pieces, washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 3. Medium in good condition; excellent growth.
	Nov. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 4. Medium in good condition; excellent growth.
	Nov. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 5. Excellent growth. Nov. 6. Medium in good condition; excellent growth (macroscopic).
	Nov. 7	Divided into 2 pieces, washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 7. Excellent growth. Nov. 8. Medium in good condition; growing.
	Nov. 9	Cut into 2 pieces, washed in Ringer solution for $\frac{1}{2}$ minute, and cultivated in the same medium	Nov. 9. Good growth. Nov. 10. Medium in good condition; excellent growth.
	Nov. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 11. Medium in good condition; excellent growth.
	Nov. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 12. Medium in good condition; growing well. Nov. 13. Medium in good condition; good growth.

TABLE I.—Continued.

Passage.	Date (1913).	Treatment of culture.	Observations.
13	Nov. 13	Culture washed in Ringer solution for 1 minute and cultivated in Nov. 15. the same medium	Nov. 14. Growing. Nov. 15. Good growth.
14	Nov. 15	Partially divided, washed in Ringer solution, and cultivated in the same medium	Nov. 17. Good growth.
15	Nov. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 18. Good growth. Nov. 19. Excellent growth.
16	Nov. 19	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Nov. 20. Medium slightly liquefied; growing. Nov. 21. More liquefaction of medium; excellent growth.
17	Nov. 21	Washed in Ringer solution for $\frac{1}{2}$ minute and cultivated in the same medium	Nov. 22. Excellent growth.
18	Nov. 22	Washed in Ringer solution for $\frac{1}{2}$ minute and cultivated in the same medium	Nov. 23. Good growth. Nov. 24. Growing.
19	Nov. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 25. Very good growth. Nov. 26. Liquefaction; excellent growth.
20	Nov. 26	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Nov. 27. Excellent growth. Nov. 28. Liquefaction; excellent growth.
21	Nov. 28	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Nov. 29. Very good growth.
22	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. Good growth. Dec. 1. Liquefaction; excellent growth.
23	Dec. 1	Washed in Ringer solution for $\frac{1}{2}$ minute and cultivated in the same medium	Dec. 2. Excellent growth (dense).
24	Dec. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 3. Good growth (dense). Dec. 4. Good growth (dense).
25	Dec. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 5. Good growth. Dec. 6. Excellent growth.
26	Dec. 6	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Dec. 7. Growing slowly. Dec. 8. Liquefaction and growing slowly. All cultures this day liquefied.
27	Dec. 8	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Dec. 9. Growing. Dec. 10. Slight liquefaction; growing.
28	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Growing. Dec. 12. Growing.
29	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth. Dec. 15. Excellent growth.

TABLE I.—Concluded.

Passage.	Date (1913).	Treatment of culture.	Observations.
30	Dec. 15	Central piece extirpated and the remaining cells cultivated in 1 drop of plasma and 1 drop of extract	Dec. 16. Excellent growth. Dec. 17. Excellent growth.
31	Dec. 17	Washed in Ringer solution and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 drop of extract	Dec. 18. Growing very well. Dec. 19. Growing very well.
32	Dec. 19	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Dec. 20. Liquefaction; growing. Dec. 21. Liquefaction; excellent growth.
33	Dec. 22	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 22. Excellent growth. Dec. 23. Growing. Dec. 24. Medium in good condition; no liquefaction. Dec. 25. Medium in good condition; no liquefaction. Dec. 26. Medium in good condition; no liquefaction.
34	Dec. 26	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Dec. 27. Growing slowly. Dec. 28. Growing slowly. Dec. 29. Liquefaction; growing slowly.
35	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. No growth; ameboid cells. Dec. 31. Complete liquefaction.
36	Dec. 31	Culture washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2, 1914. Few cells, mostly ameboid in character.

first and succeeding passages growth was more extensive in a given time than that which develops in a primitive culture; also a definite relation between the extent of growth and the degree of liquefaction which is produced by the growth was suggested.

Fluctuations were observed in the rate and extent of growth after passages, a point which is shown in the history referred to. This fluctuation affected all cultures which were changed at the same time and into the same medium. The probable causes are the differences in various specimens of plasma and the effect of the extract employed on them. In successive passages the primitive fragment gradually disappeared; and on extirpation of the primitive fragment the peripheral cells after being washed and cultivated in fresh medium continued to proliferate and form a network of tissue as shown in figure 1. Figure 2 shows the extirpated fragment which

was washed and cultivated and incubated for forty-eight hours, after which it was stained.

In actively growing cultures the cells appeared as delicate spindle-shaped, fusiform bodies, isolated or united by filaments sometimes closely assembled, forming a network which was either film-like or quite dense, depending upon the tendency of the cellular outgrowths. The cytoplasm appeared granular and the nucleus stood out as a clear, homogeneous area in which mitotic figures were distinguishable. Refractile globules were to be seen in the cytoplasm of the cells. Their size and number varied. The longer a culture was left unchanged the larger and more numerous were these globules. When conditions in the culture were unfavorable, the cells presented ameboid characteristics, and there was no tendency towards the formation of a cellular network. Refractile fat globules then became frequent in the cell cytoplasm.

When the cultures were fixed and stained with Giemsa stain the outline and structure of the cells became distinct. The cytoplasm appeared as a light blue, finely granular body with a more deeply stained, coarsely granular nucleus. The structures containing chromatin took on a deep purple stain. Figure 3 represents a photograph of stained individual cells highly magnified.

Although growth of human connective tissue derived from fetal heart tissue has been obtained which was as extensive as the growth of connective tissue derived from embryonic heart tissue of the chick, the growth of human tissue was usually less dense and less extensive. Fluctuations which occurred during the passages of human cultures were greater than those occurring in cultures of chick tissue. Liquefaction in human tissue cultures was more marked and eventually interfered with cell proliferation. In cultures of chick tissue the liquefaction that took place was slight and did not interfere with cell proliferation.

The fetal human tissue which was used in making cultures was generally obtained from fetal cadavers 4 to 6 months old; the embryonic chick tissue was obtained from 8 to 15 day old embryos. The latent period where human tissue was cultivated was usually from 16 to 18 hours. For chick tissue growth appears within 10 or 12 hours after the original fragment has been embedded.

## SUMMARY.

A strain of human connective tissue was kept in a condition of active life *in vitro* for more than two months. When a medium has been devised the composition of which is more constant, it is reasonable to suppose that human connective tissue can be cultivated *in vitro* for an indefinite period.

## EXPLANATION OF PLATES.

## PLATE 75.

FIG. 1. Human connective tissue cells, fixed and stained with Giemsa stain. The culture was made by extirpating the central portion of culture 285 in its 16th passage, washing the remaining portion of the culture with Ringer solution without removing it from the cover-glass, and dropping on fresh plasma and extract. The preparation shows the extent of growth obtained in 48 hours from peripheral cells remaining after extirpation of the fragment.

## PLATE 76.

FIG. 2. 17th passage of human connective tissue, fixed and stained with Giemsa stain. The photograph shows the extent of growth obtained after passage into fresh medium. The fragment in this culture was the piece extirpated from culture 285.

## PLATE 77.

FIG. 3. 17th passage of human connective tissue cells. Individual cells; high power magnification. Some of the cells are shown in figure 2.

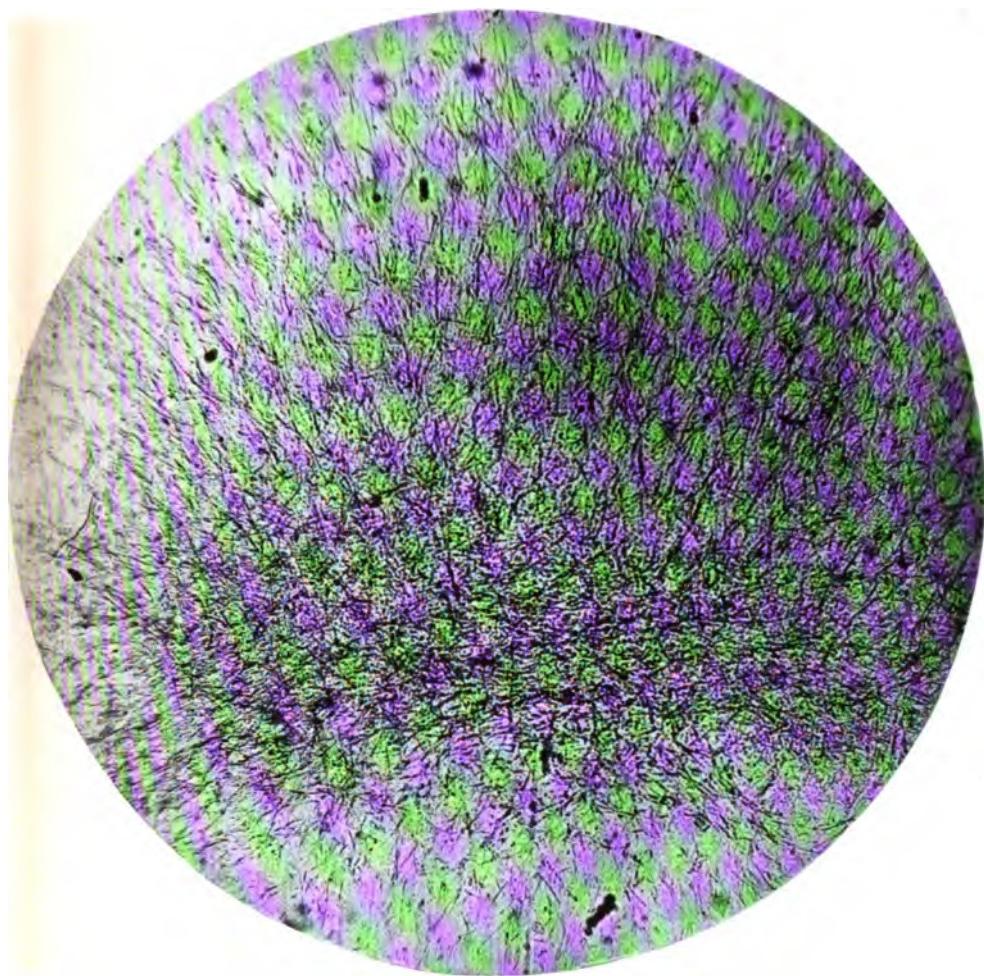


FIG. I.

Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)





FIG. 2.

(Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)





FIG. 2.

(Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)



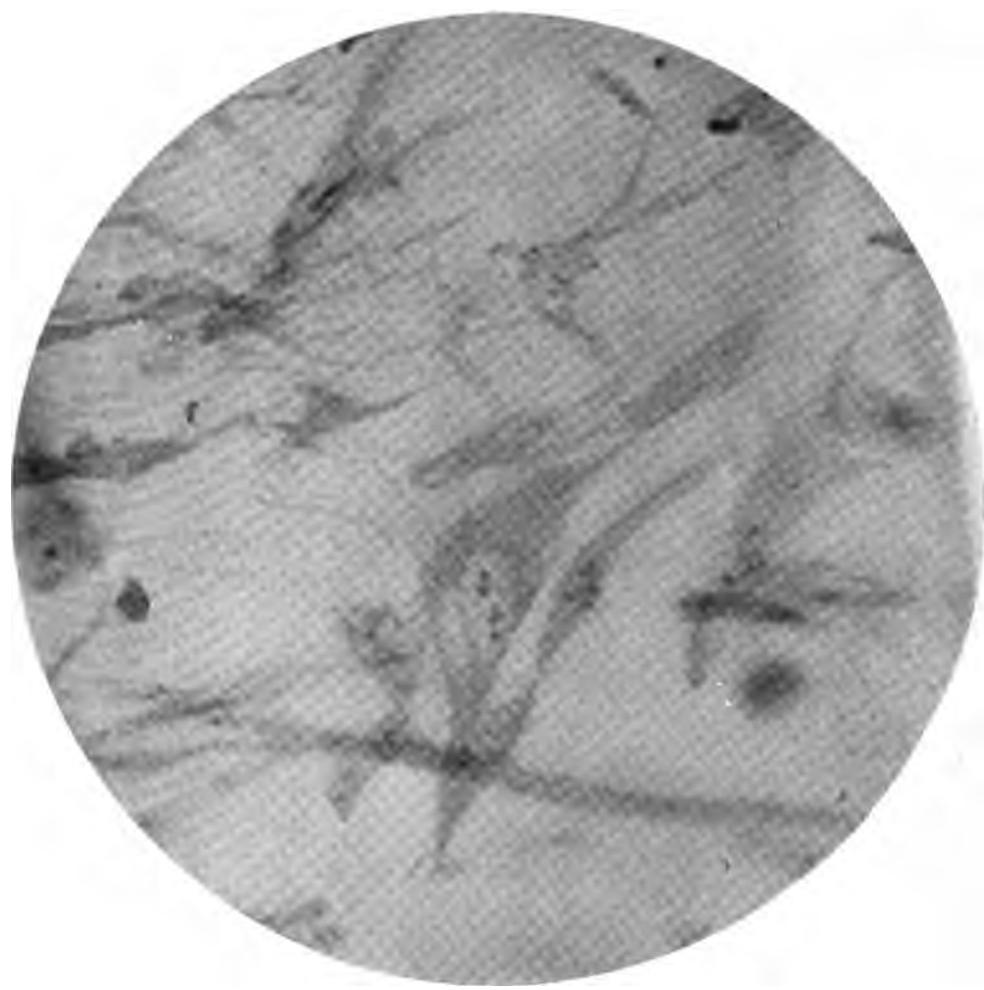


FIG. 3.

(Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)



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